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<b>(54) Title:</b> EXPRESSION OF SEDOHEPTULOSE 1,7 BISPSPHATASE IN TRANSGENIC PLANTS  <b>(57) Abstract</b>  Sedoheptulose 1,7-bisphosphatase (SBPase) is an enzyme catalyzing the reaction converting sedoheptulose 1,7-bisphosphate into sedoheptulose 7-phosphate. This enzyme is located in the chloroplast in leaves and stems. Overexpression of SBPase in transgenic plants is provided to improve plant yield by increasing leaf starch biosynthetic ability in particular and sucrose production in general. Deregulated variants of the enzymes are also provided.		

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**EXPRESSION OF SEDOHEPTULOSE 1,7 BISPHTHOSPHATASE  
IN TRANSGENIC PLANTS**

This application claims priority of the US provisional application, serial number  
5 60/133,964, filed on May 13, 1999.

**FIELD OF THE INVENTION**

This invention relates to the expression of sedoheptulose 1,7 bisphosphatase (SBPase) in transgenic plants to increase or improve plant growth and development, yield, vigor, and  
10 distribution of carbon assimilates. Transgenic plants expressing SBPase have improved carbon assimilation, export and storage in plant source and sink organs, which results in growth, yield and quality improvements in crop plants.

**BACKGROUND OF THE INVENTION**

Recent advances in genetic engineering have provided the prerequisite tools to transform  
15 plants to contain alien (often referred to as "heterogenous or heterologous") or improved endogenous genes. The introduction of such a gene in a plant would desirably lead to an improvement of an already existing pathway in plant tissues or introduction of a novel pathway to modify desired product levels, increase metabolic efficiency, and/or save on energy cost to the  
20 cell. It is presently possible to produce plants with unique physiological and biochemical traits and characteristics of high agronomic and crop processing importance. Traits that play an essential role in plant growth and development, crop yield potential and stability, and crop quality and composition are particularly desirable targets for crop plant improvement. These improvements may be achieved by genetically modifying a crop plant for improved carbon  
25 assimilation, more efficient carbon storage, and/or increased carbon export and partitioning capabilities.

Atmospheric carbon fixation (photosynthesis) by plants, algae, and photosynthetic bacteria represents the major source of energy to support processes in such organisms. The Calvin cycle, located in the stroma of the chloroplast, is the primary pathway of carbon  
30 assimilation in higher plants. Carbon assimilates can either leave the cycle for sucrose or starch biosynthesis or continue through the cycle to regenerate the carbon acceptor molecule, ribulose-1,5-bisphosphate. Sedoheptulose-1,7-bisphosphatase is an enzyme that catalyzes an essentially

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irreversible reaction in the branch region where intermediates can leave the cycle, and therefore it may be essential to regulating carbon partitioning between the regeneration phase of the cycle and sucrose and starch biosynthesis.

SBPase has no known cytosolic counterpart and is reported to be found only in the chloroplast, where it dephosphorylates sedoheptulose-1,7-bisphosphate (SBP) to form sedoheptulose-7-phosphate and inorganic phosphate. This enzyme is specific for SBP and is inhibited by its products as well as glycerate (Schimkat et al., 1990) and fructose-2,6-bisphosphate (Cadet and Meunier, 1988b). Light, a reducing agent, and  $Mg^{2+}$  are required for activity (Woodrow, 1982; Cadet and Meunier, 1988a). The enzyme is a homodimer with a subunit molecular mass of 35-38 kDa (Nishizawa and Buchanan, 1981; Cadet and Meunier, 1988c).

It has been reported that removal of more than 80% of the enzymatic activity of SBPase in tobacco plants using antisense technology resulted in chlorosis, reduced growth rates, and reduced carbon assimilate levels (Harrison et al., 1998). Reduction in the quantum efficiency of photosystem II was also observed, which resulted in the reduction in carbohydrate content of the leaves. Analysis of carbohydrate status showed a shift from starch while sucrose levels were maintained. These results indicate that SBPase is a potential rate-limiting step in carbohydrate metabolism.

Various sedoheptulose 1,7-bisphosphatases have been characterized biochemically, and the corresponding mRNAs (cDNA) have been cloned from an alga (Genbank accession number: X74418; Hahn and Kuck, 1994) and some higher plants such as *Triticum aestivum* (Genbank accession number: X65540; Miles et al., 1993), *Spinacia oleracea* (Genbank accession number: L76556; Martin et al., 1996) and *Arabidopsis thaliana* (Genbank accession number: S74719; Willingham, et al., 1994 ). Thus, over-expression of a nucleic acid sequence encoding SBPase in a transgenic plant will provide advantageous results in the plant such as improved carbon assimilation, export and storage; increased photosynthetic capacity; and extended photosynthetic ability.

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## SUMMARY OF THE INVENTION

The present invention provides a method for improving the assimilation of carbon in plants using structural nucleic acid constructs that encode a sedoheptulose 1,7-bisphosphatase (SBPase) enzyme.

5 In accomplishing the foregoing, there is provided, in accordance with one aspect of the present invention, a method for improving the assimilation of carbon in a plant comprising the steps of:

(a) inserting into the genome of a plant a nucleic acid sequence comprising in the 5' to 3' direction and operably linked,

10 (i) a promoter that functions in the cells of a selected plant tissue,

(ii) a structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme,

(iii) a 3' non-translated nucleic acid sequence that functions in plant cells to cause transcriptional termination and the addition of polyadenylated nucleotides to the  
15 3' end of a RNA sequence;

(b) obtaining transformed plant cells containing the nucleic acid of step (a); and

(c) regenerating from transformed plant cells a transformed plant that overexpresses the sedoheptulose 1,7-bisphosphatase enzyme in the plant cells.

In a further embodiment of the present invention an isolated nucleic acid sequence  
20 comprising a promoter capable of functioning in a plant cell, a structural nucleic acid sequence in sense orientation capable of causing the production of a sedoheptulose 1,7-bisphosphatase enzyme, and a 3' non-translated nucleic acid sequence capable of causing transcriptional termination and the addition of polyadenylated nucleotides to the 3' end of the transcribed mRNA sequence, is provided. This nucleic acid sequence may optionally include introns, 5'  
25 untranslated leader sequences or other nucleic acid sequences designed to enhance transcription and/or translation.

In a still further embodiment of the invention, a novel, isolated nucleic acid sequence encoding a sedoheptulose 1,7-bisphosphatase enzyme from a green algae, *Chlorella sorokiniana*, is provided.

30 In a yet further embodiment of the present invention, a variant nucleic acid sequence encoding a sedoheptulose 1,7-bisphosphatase enzyme is provided whereby the cysteine residues

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in the polypeptide sequence are modified to another amino acid in a manner providing an active enzyme regardless of the presence of light.

Therefore, in accordance with the present invention, a means for improving carbon assimilation, storage and export in the source tissues of a plant is provided. Further means of improved carbon accumulation in sinks (such as roots, tubers, seeds, stems, and bulbs) are provided, thus increasing the size of various sinks (larger roots and tubers) and subsequently increasing yield. The increased carbon availability to these sinks would improve and/or alter the composition of the cellular components of the plant (e.g., oils, proteins, starch and sucrose production and solids uniformity). One aim of the present invention is to overexpress sedoheptulose 1,7-bisphosphatases in plants by introducing a heterologous source of the sedoheptulose 1,7-bisphosphatase into the plant or by increasing the expression of the endogenous form of the gene in the plant.

Various advantages may be achieved by the aims of the present invention. Increasing the expression of the sedoheptulose 1,7-bisphosphatase enzyme in the chloroplast would increase the flow of carbon through the Calvin Cycle and potentially increase atmospheric carbon assimilation in the presence of light. This would result in an increase in photosynthetic efficiency, an increase in chloroplast starch production (a leaf carbon storage form degraded during periods when photosynthesis is low or absent), and an increase in sucrose production by the leaf resulting in a net increase in carbon export to the sink and developing tissues would be expected during a given photoperiod. This increase in source capacity is a desirable trait in crop plants and would lead to increased plant growth, storage ability, yield and vigor.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1** shows a plasmid map for cloning vector pMON47205.
- Figure 2** shows a plasmid map for cloning vector pMON47207.
- Figure 3** shows a plasmid map for cloning vector pMON47208.
- Figure 4** shows a plasmid map for plant transformation vector pMON10098.
- Figure 5** shows a plasmid map for plant transformation vector pMON47200.
- Figure 6** shows a plasmid map for shuttle vector pMON999.
- Figure 7** shows a plasmid map for transient transformation vector pMON47203.

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**Figure 8** shows an immunoblot of SBPase protein expression in corn protoplasts transformed with pMON47203.

**Figure 9** shows a bar graph comparing SBPase activity in corn protoplasts expressing wheat SBPase with control protoplasts.

5 **Figure 10** shows an alignment of Sedoheptulose-1,7-bisphosphatase proteins.

### DESCRIPTION OF SEQUENCES

- SEQ ID NO:1 synthetic primer
- SEQ ID NO:2 synthetic primer
- 10 SEQ ID NO:3 DNA sequence for mature SBPase (no CTP)
- SEQ ID NO:4 synthetic primer
- SEQ ID NO:5 adaptor primer
- SEQ ID NO:6 synthetic primer
- SEQ ID NO:7 adaptor primer
- 15 SEQ ID NO:8 DNA sequence of SBPase with CTP
- SEQ ID NO:9 amino acid sequence of SBPase
- SEQ ID NO:10 a degenerate primer
- SEQ ID NO:11 a gene-specific primer
- SEQ ID NO:12 a predicted amino acid sequence
- 20 SEQ ID NO:13 a gene-specific primer
- SEQ ID NO:14 a nested gene-specific primer
- SEQ ID NO:15 a gene-specific primer
- SEQ ID NO:16 a gene-specific primer
- SEQ ID NO:17 a nested gene-specific primer
- 25 SEQ ID NO:18 a gene-specific primer
- SEQ ID NO:19 a vector-specific primer
- SEQ ID NO:20 the full length *Chlorella sorokiniana* SBPase cDNA sequence
- SEQ ID NO:21 a mutagenic primer to change cysteines 110 and 115 to serines in *Chlorella sorokiniana* SBPase.
- 30 SEQ ID NO:22 a variant cDNA sequence in which cysteines 110 and 115 of *Chlorella sorokiniana* SBPase are changed to serines.

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SEQ ID NO: 23 a predicted variant protein sequence of *Chlorella sorokiniana* SBPase in which cysteines 110 and 115 are changed to serines.

### DETAILED DESCRIPTION OF THE INVENTION

5 The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

The term "identity" refers to amino acid or nucleic acid sequences that when compared using the *local homology* algorithm of Smith and Waterman (1981)) in the BestFit program (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc.) are  
10 exactly alike.

The term "similarity" refers to amino acid sequences that when compared using the *local homology* algorithm of Smith and Waterman (1981) in the BestFit program (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc.) match when conservative amino acid substitutions are considered.

15 "C-terminal region" refers to the region of a peptide, polypeptide, or protein chain from the middle thereof to the end that carries the amino acid having a free carboxyl group. The phrase "nucleic acid or DNA segment heterologous to the promoter region" means that the coding DNA or nucleic acid segment does not exist in nature with the promoter to which it is now operably linked or coupled therewith.

20 The term "encoding DNA" refers to chromosomal DNA, plasmid DNA, cDNA, or synthetic DNA that encodes any of the enzymes discussed herein.

The term "genome" as it applies to bacteria encompasses both the chromosome and plasmids within a bacterial host cell. Encoding DNAs of the present invention introduced into bacterial host cells can therefore be either chromosomally integrated or plasmid-localized. The  
25 term "genome" as it applies to plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components of the cell. DNAs of the present invention introduced into plant cells can therefore be either chromosomally integrated or organelle-localized.

The terms "microbe" or "microorganism" refer to algae, bacteria, fungi, and protozoa.

30 The term "mutein" refers to a mutant form of a peptide, polypeptide, or protein.



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"N-terminal region" refers to the region of a peptide, polypeptide, or protein chain from the amino acid having a free amino group to the middle of the chain.

"Overexpression" refers to the expression of a polypeptide or protein encoded by a DNA introduced into a host cell, wherein said polypeptide or protein is either not normally present in the host cell, or wherein said polypeptide or protein is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding said polypeptide or protein.

The term "plastid" refers to the class of plant cell organelles that includes amyloplasts, chloroplasts, chromoplasts, elaioplasts, eoplasts, etioplasts, leucoplasts, and proplastids. These organelles are self-replicating, and contain what is commonly referred to as the "chloroplast genome," a circular DNA molecule that ranges in size from about 120 to about 217 kb, depending upon the plant species, and which usually contains an inverted repeat region.

This invention is directed to a method for producing plant cells demonstrating an increased or improved plant growth and development, yield and vigor. The method utilizes a DNA sequence encoding an *sbpase* (sedoheptulose 1,7-bisphosphatase) gene integrated in the cellular genome of a plant as the result of genetic engineering and causes expression of the SBPase enzyme. Plants that overexpress the SBPase enzyme and that have improved carbon assimilation, export and storage in plant source and sink organs, which results in growth, yield, and quality improvements are also contemplated in this invention.

The mechanism whereby the expression of exogenous SBPase modifies carbon relationships derives from source-sink relationships. The leaf tissue is a sucrose source, and if more sucrose, resulting from the activity of increased SBPase expression, is transported to a sink it results in increased storage carbon (sugars, starch, etc.) per given weight of the sink tissue.

A method for producing genetically transformed plants that express increased levels of SBPase requires the introduction of a double-stranded recombinant DNA molecule into the nuclear genome of a plant cell. The DNA molecule must (1) contain a structural DNA for the SBPase enzyme being introduced into the plant cell; (2) possess a promoter that functions in plants to regulate the production of an RNA sequence in a constitutive or tissue-specific manner by RNA polymerase enzyme; and (3) have a 3'-untranslated region that functions to cause transcriptional termination and the addition of polyadenylated nucleotides to the 3' end of the RNA. The resulting primary RNA molecule is subsequently processed in the nucleus, a process

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that involves the removal of intronic sequences and the addition of polyadenylate nucleotides to the 3' end of the mRNA.

Sedoheptulose 1,7-bisphosphatases

As used herein, the term "sedoheptulose 1,7-bisphosphatase" means an enzyme (E.C. 3.1.3.37) that catalyzes the dephosphorylation of sedoheptulose 1,7-bisphosphate to sedoheptulose 7-phosphate and inorganic phosphate.

The SBPase gene used in the DNA constructs of the present invention can be any SBPase gene. Numerous SBPase cDNA sequences are known in the art including the sequences from wheat (Raines et al., 1992), spinach (Martin et al., 1996), *Arabidopsis* (Willingham et al., 1994), and *Ralstonia* (Yoo and Bowien, 1995). The examples provided herein are illustrative of the use of an SBPase, and should not be interpreted in any way to limit the scope of the present invention. Individuals skilled in the art will recognize that various other genes as well as alterations can be made to genes and methods described herein while not departing from the spirit and scope of the present invention. For example, one could utilize a SBPase that has been selected from alternative organisms or that has been modified to lack or alter enzymatic feedback inhibition by orthophosphate and glycerate. Because SBPase is activated by thioredoxin or DTT, one could also use mutagenesis to manipulate the enzyme to remain in the activated state without the reducer present. These mutated forms can then be utilized to modify plant metabolism as well. The overproduction of carbohydrate may also provide an increase in tolerance to stresses that affect the water potential of the plants by providing more carbon skeletons. Further means of improved carbon accumulation in sinks (such as roots, tubers, seeds, stems, and bulbs) are provided, thus increasing the size of various sinks (larger roots and tubers) and subsequently increasing yield. The increased carbon availability to these sinks would improve cellular component composition (e.g., oil, protein, starch and sucrose production and solids uniformity). Thus, many different nucleic acid sequences that encode a sedoheptulose 1,7-bisphosphatase activity may be isolated and used in the present invention.

The SBPase cDNA from *Chlorella sorokiniana* is identified herein and may advantageously be used in connection with this invention. This gene may also be deregulated with respect to light by alteration of the cysteine residues to another amino acid, e.g., serine. Preferably, any cDNA sequence encoding SBPase that is about 85% identical to the *Chlorella sorokiniana* cDNA is within the scope of this invention, more preferably such cDNA sequence

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that is about 90% identical to the *Chlorella sorokiniana* cDNA, and most preferably about 95% identical to the *Chlorella sorokiniana* cDNA. Moreover, any amino acid sequence encoding SBPase that is about 85%, and more preferably about 90% similar to the *Chlorella sorokiniana* predicted amino acid sequence disclosed herein is considered within the scope of this invention.

5        Bisphosphatase genes from the facultative chemoautotroph *Alcaligenes eutrophus* (now named *Ralstonia eutropha*) and from the algae *Synechococcus lepoliensis* encode proteins with a dual activity, FBPase and SBPase, in the Calvin cycle (Yoo and Bowien, 1995; Gerbling et al., 1986). Genes encoding proteins with SBPase activity can be cloned from these or other organisms by anyone skilled in the art using well established methods. The source of the SBPase  
10        genes for use in this invention as described is not limited to the organisms described above and should not be interpreted in any way to limit the scope of the present invention. In one embodiment of the invention, the SBPase gene may be fused to a chloroplast transit peptide, in order to target the SBPase protein to the plastid. Those skilled in the art will also recognize that various other chimeric constructs can be made that utilize the functionality of a particular plastid  
15        transit peptide to import the sedoheptulose-1,7-bisphosphatase enzyme into the plant cell plastid depending on the promoter tissue specificity.

#### Gene construction and modifications

A sedoheptulose 1,7 bisphosphatase enzyme considered in this invention includes any sequence of amino acids, such as protein, polypeptide, or peptide fragment, that demonstrates the  
20        ability to catalyze the dephosphorylation of sedoheptulose 1,7-bisphosphate to sedoheptulose 7-phosphate and inorganic phosphate. As described above, these can be sequences obtained from a heterologous source, such as algae, bacteria, fungi, and protozoa, or endogenous plant sequences, by which is meant any sequence that can be naturally found, in a plant cell, including native (indigenous) plant sequences as well as sequences from plant viruses or plant pathogenic  
25        bacteria.

It will be recognized by one of ordinary skill in the art that SBPase enzyme gene sequences may also be modified using standard techniques such as site-specific mutation or PCR, or modification of the sequence may be accomplished by producing a synthetic nucleic acid sequence and will still be considered a SBPase enzyme nucleic acid sequence of this  
30        invention. For example, wobble positions in codons may be changed such that the nucleic acid sequence encodes the same amino acid sequence, or alternatively, codons can be altered such

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that conservative or nonconservative amino acid substitutions result. In either case, the peptide or protein maintains the desired enzymatic activity and is thus considered part of this invention.

In one embodiment of the invention, the SBPase nucleic acid sequence is modified to change cysteine residues in the amino acid sequence to a different amino acid to prevent formation of disulfide bridges in the mature polypeptide between the cysteine residues providing an active enzyme regardless of the presence of light and also therefore prevent inactivation of the native protein by oxidation. For example, in the wheat SBPase, the cysteine residues at positions 52 and 57 (numbers correspond to mature wheat SBPase as described in Raines et al. (1999)) are modified to a different amino acid such as a serine, alanine or glycine to prevent formation of the disulfide bond there between. Correspondingly, the cysteine residues at amino acid positions 110 and 115 of the *Chlorella* SBPase (corresponding to the *Chlorella* SBPase numbering in SEQ ID NO:12) would also be modified for the same effect.

A nucleic acid sequence to a SBPase enzyme may be a DNA or RNA sequence, derived from genomic DNA, cDNA, mRNA, or may be synthesized in whole or in part. The structural gene sequence may be cloned, for example, by isolating genomic DNA from an appropriate source, and amplifying and cloning the sequence of interest using a polymerase chain reaction (PCR). Alternatively, the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired structural gene may be synthesized using codons preferred by a selected plant host. Plant-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular plant host species. Other modifications of the gene sequences may result in mutants having slightly altered activity.

If desired, the gene sequence of the *sbpase* gene can be changed without changing the protein/amino acid sequence in such a manner as may increase expression and thus even more positively affect carbohydrate content in transformed plants. A preferred manner for making the changes in the *sbpase* gene sequence are set out in PCT Publication WO 90/10076. A gene synthesized by following the methodology set out therein may be introduced into plants as described below and result in higher levels of expression of the SBPase enzyme. This may be particularly useful in monocots such as maize, rice, wheat, sugarcane and barley.

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### Promoters

A number of promoters that are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S and the figwort mosaic virus (FMV) 35S-promoters, the light-inducible promoter from the small subunit of ribulose-1,5-bisphosphate carboxylase (ssRUBISCO), a very abundant plant polypeptide, and the chlorophyll a/b binding protein gene promoters, etc. All of these promoters have been used to create various types of DNA constructs that have been expressed in plants; see, e.g., PCT publication WO 84/02913.

Promoters that are known to or are found to cause transcription of DNA in plant cells can be used in the present invention. Such promoters may be obtained from a variety of sources such as plants and plant viruses and include, but are not limited to, the enhanced CaMV35S promoter and promoters isolated from plant genes such as ssRUBISCO genes. It is preferred that the particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of sedoheptulose 1,7-bisphosphatase enzyme to cause the desired increase in carbon assimilation, export and storage. Expression of the double-stranded DNA molecules of the present invention can be driven by a constitutive promoter, expressing the DNA molecule in all or most of the tissues of the plant. In addition, it may also be preferred to bring about expression of the *sbpase* gene in specific tissues of the plant, such as leaf or stem, and the promoter chosen should have the desired tissue and developmental specificity. Those skilled in the art will recognize that the amount of sedoheptulose 1,7-bisphosphatase needed to induce the desired increase in carbon assimilation, export, or storage may vary with the type of plant. Therefore, promoter function should be optimized by selecting a promoter with the desired tissue expression capabilities and approximate promoter strength and selecting a transformant that produces the desired sedoheptulose 1,7-bisphosphatase activity or the desired change in metabolism of carbohydrates in the target tissues. This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants because there is variation between transformants containing the same heterologous gene due to the site of gene insertion within the plant genome (commonly referred to as "position effect"). In addition to promoters that are known to cause transcription (constitutively or tissue-specific) of

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DNA in plant cells, other promoters may be identified for use in the current invention by screening a plant cDNA library for genes that are selectively or preferably expressed in the target tissues and then determine the promoter regions.

For the purpose of expressing the *sbpase* gene in source tissues of the plant, such as the leaf or stem, it is preferred that the promoters utilized in the double-stranded DNA molecules of the present invention have increased expression in these specific tissues. For this purpose, one may choose from a number of promoters for genes with leaf-specific or leaf-enhanced expression. Examples of such genes known from the literature are the chloroplast glutamine synthetase GS2 from pea (Edwards et al., 1990), the chloroplast fructose-1,6-bisphosphatase (FBPase) from wheat (Lloyd et al., 1991), the nuclear photosynthetic ST-LS1 from potato (Stockhaus et al., 1989), and the phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) genes from *Arabidopsis thaliana* (Leyva et al., 1995). Also shown to be active in photosynthetically active tissues are the ribulose-1,5-bisphosphate carboxylase (RUBISCO) isolated from eastern larch (*Larix laricina*) (Campbell et al., 1994); the *cab* gene, encoding the chlorophyll a/b-binding protein of PSII, isolated from pine (*cab6*; Yamamoto et al., 1994), wheat (*Cab-1*; Fejes et al., 1990), spinach (*CAB-1*; Luebbert et al., 1994), and rice (*cab1R*; Luan et al., 1992); the pyruvate orthophosphate dikinase (PPDK) from maize (Matsuoka et al., 1993); the tobacco *Lhcb1\*2* gene (Cerdan et al., 1997); the *Arabidopsis thaliana* *SUC2* sucrose-H<sup>+</sup> symporter gene (Truernit et al., 1995); and the thylacoid membrane proteins, isolated from spinach (*psaD*, *psaF*, *psaE*, *PC*, *FNR*, *atpC*, *atpD*, *cab*, *rbcS*; Oelmueller et al., 1992). Other chlorophyll a/b-binding proteins have been studied and described in the literature, such as *Lhcb* and *PsbP* from white mustard (*Sinapis alba*; Kretsch et al., 1995). Promoters that cause the production of SBPase specifically in the stems, leaves, or specific cell types in these tissues are useful in the present invention. For example, the *RbcS* bundle sheath-specific promoter is one such tissue-specific promoter. Thus native promoters for maize, wheat, barley, and rice may be obtained and used in the present invention as well as heterologous promoters from other organisms shown to function in a constitutive/tissue-specific manner. Carbon metabolism in C4 plants such as corn is more specialized than in C3 plants such as tobacco. In C4 plants, metabolites generated by the Calvin cycle in the chloroplast of bundle sheath cells must be transported to the cytoplasm of the mesophyll cells where sucrose biosynthesis takes place. Therefore, cell-specific promoters are needed for correct gene expression in the proper cell type

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in C4 crops. For example, the RbcS bundle sheath-specific promoter would be useful for expressing SBPase in the appropriate cell type in corn.

For the purpose of expressing an *sbpase* gene (encoding a light-regulated protein or a protein modified to be constitutively active) only when the plant is photosynthetically active, it is preferred that the promoters utilized in the double-stranded DNA molecules of the present invention have expression in the presence of light only. For this purpose, one may choose from a number of promoters for light regulated genes, including FBPase from wheat (Miles, et al., 1993), pyruvate orthophosphate dikinase from maize (Sheen, 1991), and chloroplast aldolase from rice (Kagaya et al., 1995).

The RNA produced by a DNA construct of the present invention may also contain a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs, as presented in the following examples, wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence.

Generally, optimal expression in monocotyledonous and some dicotyledonous plants is obtained when an intron sequence is inserted between the promoter sequence and the structural gene sequence or, optionally, may be inserted in the structural coding sequence to provide an interrupted coding sequence. An example of such an intron sequence is the HSP 70 intron described in WO 93/19189.

#### Polyadenylation signal

The 3' non-translated region of the chimeric plant gene contains a polyadenylation signal that functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes like the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene.

#### Plastid-directed expression of sedoheptulose 1,7-bisphosphatase activity

In one embodiment of the invention, the *sbpase* gene may be fused to a chloroplast transit peptide, in order to target the SBPase protein to the plastid. As used hereinafter, chloroplast and plastid are intended to include the various forms of plastids including amyloplasts. Many plastid-localized proteins are expressed from nuclear genes as precursors and are targeted to the plastid by a chloroplast transit peptide (CTP), which is removed during the import steps. Examples of such chloroplast proteins include the small subunit of ribulose-1,5-bisphosphate carboxylase (ssRUBISCO, SSU), 5-enolpyruvateshikimate-3-phosphate synthase (EPSPS), ferredoxin, ferredoxin oxidoreductase, the light-harvesting-complex protein I and protein II, and thioredoxin F. The plastid targeting sequence can be, but is not limited to, the native chloroplast targeting peptide (CTP) identified in the wheat SBPase cDNA. It has been demonstrated that non-plastid proteins may be targeted to the chloroplast by use of protein fusions with a CTP and that a CTP sequence is sufficient to target a protein to the plastid. Those skilled in the art will also recognize that various other chimeric constructs can be made that utilize the functionality of a particular plastid transit peptide to import the enzyme into the plant cell plastid depending on the promoter tissue specificity.

#### Combinations with other transgenes

The effect of *sbpase* in transgenic plants may be enhanced by combining it with other genes that positively affect carbohydrate assimilation or content, such as a gene encoding for a sucrose phosphorylase as described in PCT Publication WO 96/24679, or ADPGPP genes such as the *E. coli glgC* gene and its mutant *glgC16*. PCT Publication WO 91/19806 discloses how to incorporate the latter gene into many plant species in order to increase starch or solids. Another gene that can be combined with *sbpase* to increase carbon assimilation, export or storage is a gene encoding for sucrose phosphate synthase (SPS). PCT Publication WO 92/16631 discloses one such gene and its use in transgenic plants. Another gene with which SBPase can be combined is fructose 1,6-bisphosphate aldolase.

#### Plant transformation/regeneration

In developing the nucleic acid constructs of this invention, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector, e.g., a plasmid that is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature, many of which are commercially available. After each



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cloning, the cloning vector with the desired insert may be isolated and subjected to further manipulation, such as restriction digestion, insertion of new fragments or nucleotides, ligation, deletion, mutation, resection, etc. so as to tailor the components of the desired sequence. Once the construct has been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

A double-stranded DNA molecule of the present invention containing an *sbpase* gene can be inserted into the genome of a plant by any suitable method. Preferred methods of transformation of plant cells or tissue are the *Agrobacterium* mediated transformation method and the biolistics or particle-gun mediated transformation method. Suitable plant transformation vectors for the purpose of *Agrobacterium* mediated transformation include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella et al. (1983), Bevan (1984), Klee et al. (1985) and EPO publication 120,516. In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, but are not limited to, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using viruses or pollen.

A plasmid expression vector suitable for the introduction of a *sbpase* gene in monocots using electroporation or particle-gun mediated transformation is composed of the following: a promoter that is constitutive or tissue-specific; an intron that provides a splice site to facilitate expression of the gene, such as the Hsp70 intron (PCT Publication WO93/19189); and a 3' polyadenylation sequence such as the nopaline synthase 3' sequence (NOS 3'; Fraley et al., 1983). This expression cassette may be assembled on high copy replicons suitable for the production of large quantities of DNA.

An example of a useful Ti plasmid cassette vector for plant transformation is pMON-17227. This vector is described in PCT Publication WO 92/04449 and contains a gene encoding an enzyme conferring glyphosate resistance (denominated CP4), which is an excellent selection marker gene for many plants. The gene is fused to the *Arabidopsis* EPSPS chloroplast transit peptide (CTP2) and expressed from the FMV promoter as described therein. When adequate numbers of cells (or protoplasts) containing the sedoheptulose-1,7-bisphosphatase gene or cDNA are obtained, the cells (or protoplasts) are regenerated into whole plants. Choice of methodology

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for the regeneration step is not critical, with suitable protocols being available for hosts from Leguminosae (alfalfa, soybean, clover, etc.), Umbelliferae (carrot, celery, parsnip), Cruciferae (cabbage, radish, canola/rapeseed, etc.), Cucurbitaceae (melons and cucumber), Gramineae (wheat, barley, rice, maize, etc.), Solanaceae (potato, tobacco, tomato, peppers), various floral  
5 crops, such as sunflower, and nut-bearing trees, such as almonds, cashews, walnuts, and pecans. See, for example, Ammirato et al. (1984); Shimamoto et al. (1989); Fromm (1990); Vasil et al. (1990); Vasil et al. (1992); Hayashimoto (1990); and Datta et al. (1990).

Plants that can be made to have enhanced and/or improved carbon assimilation, increased carbon export and partitioning by practice of the present invention include, but are not limited to,  
10 Acacia, alfalfa, aneth, apple, apricot, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, cauliflower, celery, cherry, cilantro, citrus, clementines, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, mango, melon, mushroom, nut,  
15 oat, okra, onion, orange, an ornamental plant, papaya, parsley, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, turf, a vine, watermelon, wheat, yams, and zucchini.

20 The following examples are provided to better elucidate the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications, truncations, etc., can be made to the methods and genes described herein while not departing from the spirit and scope of the present invention.

25

## EXAMPLES

### EXAMPLE 1

#### *cDNA cloning and overexpression of SBPase for antibody production*

To isolate the region of the gene encoding the mature SBPase protein (no CTP), an RT-  
30 PCR reaction was performed. One microgram of *Triticum aestivum*, CV OSLO leaf RNA was combined with 100 pmol of random hexamer primers (BRL/Life Technologies Inc,

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Gaithersburg, MD) or with 100 pmol of oligo dT primer (Promega, Madison, WI), heated for 5 minutes at 75°C, and chilled on ice. First strand cDNA synthesis was performed using Superscript II<sup>TM</sup> reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) according to the manufacturer's protocol. The terminated reverse transcription reaction was diluted 1:7.

5 Three microliters of the diluted first strand synthesis products were combined with 100 µM of each dNTP, 50 pmol of a gene specific primer with homology to the 5' end of the gene designed to generate an *NdeI* cleavage site for subcloning purposes (5'-ACATATGTGCGCGATCGGCGA-3', SEQ ID NO:1), 50 pmol of a gene specific primer with homology to the 3' end of the gene (5'-GGATCCAGAAGAAGATTATTAGGCG-3', SEQ ID

10 NO:2), and 5 Units of PWO<sup>TM</sup> polymerase (Boehringer, Mannheim, Germany) in 100 µL. PCR cycling conditions were as follows: 95°C, 40 seconds; 56°C, 1 minute; 72°C, 1 minute 30 seconds (5 cycles) followed by 95°C, 40 seconds; 61°C, 1 minute; 72°C, 1 minute 30 seconds (30 cycles). The 982 bp SBPase mature protein gene PCR product was gel purified, cloned into the PCR-Blunt cloning vector (Invitrogen, Carlsbad, CA) to form pMON47205 (Figure 1), and

15 transformed into competent *E. coli* cells. Clones bearing inserts were selected on media containing kanamycin, plasmid purified, and digested with *BamHI* to select for the proper orientation in the cloning vector. Sequence analysis revealed that predicted amino acid sequence (SEQ ID NO:9) is identical to the published sequence (Raines et al., 1992) starting at residue 73, although the nucleotide sequence (SEQ ID NO:3) differs at 2 positions (residue 294 from T to C;

20 residue 572 from C to T). The selected plasmids were restricted with *NdeI* and *BamHI*, directionally cloned under the control of the IPTG inducible T7 polymerase promoter of pET 15b bacterial expression vector (Novagen, Madison, WI) linearized with *NdeI/BamHI*, and transformed into DH5α. Transformants were screened by *NdeI/BamHI* restriction analysis and clones possessing an insert were selected, plasmid purified, and transformed into *E. coli*

25 BL21(DE3) for protein expression purposes.

*E. coli* BL21(DE3) cells transformed with the pET15b-SBPase cDNA construct were induced with 2 mM IPTG for 2.5 hours after which a distinct protein band of about 38 kDa was apparent on an SDS-PAGE gel, which correlates with the size of the subunit polypeptide chain of the dimeric SBPase. Protein expressed by the mature SBPase was purified based on the affinity

30 of histidine residues for immobilized nickel ions. The purification was performed under denaturing conditions using Ni-NTA Superflow resin (QIAGEN, Valencia, CA) as described in

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the manufacturer's protocol. A 2 mL fraction containing 1 mg of purified SBPase protein emulsified with an equal volume of Complete Adjuvant (Sigma, St. Louis, MO) was used to inoculate a goat for antibody production using standard methods (Antech Company, St. Louis, MO). The preimmune serum showed no reactivity with SBPase.

## 5 EXAMPLE 2

### *cDNA cloning of SBPase for expression in plants*

To clone the region of the SBPase gene encoding the chloroplast transit peptide, a modified anchored PCR procedure for the rapid amplification of cDNA ends (Frohman, 1990; Jain et al., 1992) was used. Eight hundred nanograms of total RNA was combined with 10 ng of  
10 a gene-specific primer (5'- TTCCTCAGAGCACGCGTACTTG-3' , SEQ ID NO:4), heated to 75°C for 5 minutes, and chilled on ice. First strand DNA synthesis was performed using Superscript II<sup>TM</sup> reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) according to the supplier's protocol. The terminated reverse transcription reaction was treated with one unit of ribonuclease H for 20 minutes at 37°C, 5 minutes at 95°C, and chilled on ice. Excess  
15 primers and dNTPs were removed by centrifugation at 2,000 x g through an Ultrafree-MC filterfuge (30,000 MW cutoff, Millipore, Bedford, MA), and the retentate was concentrated to 15 µL on a Savant Speedvac (Savant Instruments, Holbrook, NY). The first-strand synthesis products were combined with 10 µL of tailing mix (1X tailing buffer [BRL/Life Technologies Inc, Gaithersburg, MD], 0.4 mM dATP, 10 units of terminal deoxytransferase) and incubated at  
20 37°C for 10 minutes. The reaction mixture was heated to 95°C for 5 minutes, diluted to 0.5 mL with TE, pH 8.0, and utilized as a cDNA pool. A mixture of 10 µL of the cDNA pool, 10 µL PWO<sup>TM</sup> polymerase 10X buffer (BRL/Life Technologies Inc, Gaithersburg, MD), 100 µM of each dNTP, 25 pmol of a gene specific primer (SEQ ID NO:4), 10 pmol of the poly(dT) adaptor primer (5'-GGGTCGACATTCTAGACAGAATTCGTGGATCC(T)<sub>21</sub>-3'; SEQ ID NO:5), and 5  
25 units of PWO<sup>TM</sup> polymerase (BRL/Life Technologies Inc, Gaithersburg, MD) in 100 µL was amplified. PCR cycling conditions were as follows: 95°C, 2 minutes; 45°C, 5 minutes; 72°C, 40 minutes (1 cycle) followed by 95°C, 50 seconds; 48°C, 1 minute; 72°C, 1 minute (3 cycles). The PCR products were purified away from the excess primers by ethanol precipitation. The pellet was resuspended in 50 µL of water and subjected to another round of amplification using a new  
30 nested gene specific primer (5'-CATGGGAGTACTCCAACGCCTC-3', SEQ ID NO:6) and an

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adaptor primer (5'-GGGTCGACATTCTAGACAGAA-3', SEQ ID NO:7). PCR cycling conditions were as follows: 95°C, 40 seconds; 58°C, 1 minute; 72°C, 30 seconds (30 cycles). The ~500 bp PCR product was gel purified, subcloned into the PCR-Blunt cloning vector (Invitrogen, Carlsbad, CA) to form pMON47207 (Figure 2), and transformed into One Shot Top

5 10 Cells (Invitrogen, Carlsbad, CA) for further characterization. Sequence analysis (SEQ ID NO:8) showed that this sequence differs from the published sequence in 9 positions (30, C to T; 42, G to C; 44, A to G; 187, C to A; 204, C to G; 228, C to G; 259, C to T; 348, G to C; and 351, C to G) resulting in three predicted amino acid changes (30, Arg to Cys; 44, His to Arg; 207, Gln to Glu).

10 In order to test the expression of the wheat SBPase in plants, pMON47205 (Figure 1) and pMON47207 (Figure 2) were each restricted with *Sall* and *BamHI*. The fragments containing sequence encoding the mature SBPase and the CTP region, respectively, were gel purified, and ligated together to form pMON47208 (Figure 3). pMON47208 was restricted with *XbaI* and *BamHI* and the sequence encoding SBPase was gel purified and ligated to *XbaI/BamHI* -

15 linearized pMON10098 (Figure 4). The final vector formed was pMON47200 (Figure 5) with the CaMV E35S promoter, the coding sequence of the entire SBPase gene, the NOS 3'-untranslated polyadenylation region, and kanamycin resistance for selection in plants.

### EXAMPLE 3

#### *Transient expression of maize protoplasts*

20 In order to test the expression of the wheat SBPase subunits and their assembly into active enzymes, vectors were constructed to contain the CaMV E35S promoter, the coding sequence for the entire SBPase protein including the CTP, the NOS 3' termination signal, and ampicillin resistance for selection in *E. coli*. The SBPase gene was isolated as an *XbaI-BamHI* fragment from pMON47200. The SBPase gene was ligated into the *XbaI-BamHI* CaMV E35S,

25 NOS 3' bearing region of pMON999 (Figure 6) to give pMON47203 (Figure 7). The DNA constructs were electroporated into maize protoplasts according to the method of Sheen et al. (1991).

### EXAMPLE 4

#### *Analysis of transformed maize protoplasts*

30 Pelleted protoplast samples transformed with pMON47203 (SBPase) and no DNA were thawed in 0.18 mL of extraction buffer (50 mM HEPES pH 7.5, 1 mM fructose bisphosphate, 1

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mM sedoheptulose-1,7-bisphosphate, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 10 mM DTT, 1% polyvinylpolypyrrolidone, 10% glycerol, and Complete<sup>TM</sup> Protease Inhibitors (Boehringer, Mannheim, Germany)) on ice. The cells in each suspension were vortexed well and clarified at 2,000 x g for 15 minutes. The supernatants were desalted using G25 spin columns (The Nest Group, Southboro, MA). Total protein content of the desalted protein was determined using the  
5 BioRad microprotein assay (BioRad, Hercules, CA) according to the manufacturer's protocol.

Protein expression and size were determined by Western blot analysis (Figure 8). Significantly more protein was detected by cross-reactivity with goat anti-wheat SBPase antibodies in protoplasts transformed with pMON47203 than control protoplasts, indicating  
10 successful over-expression of SBPase enzyme in plant cells. The mobility of the wheat SBPase (pMON47203) expressed in corn protoplasts was approximately 38 kDa, the same as the endogenous wheat leaf SBPase, indicating correct processing of the CTP.

SBPase activity was assayed by allowing hydrolysis of SBP for 10 minutes followed by measurement of phosphate liberation. The reaction was initiated by combining 20 µL of buffer  
15 (100 mM Tris, 8.2, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1.5 mM EDTA, 10% glycerol) containing 5 µg of desalted protein extract with 55 µL of assay buffer (50 mM Tris, 8.2, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1.5 mM EDTA, 0.27 mM sedoheptulose-1,7-bisphosphate). The reaction was stopped after a 10 minute incubation at room temperature by adding 30 µL of 1 M perchloric acid. The precipitated protein was removed by centrifugation, 250 µL of developer (1% ammonium  
20 molybdate, 1N HCl, 0.05% Malachite green (Itaya and Michio, 1966)) was added to 50 µL of supernatant, and the amount of phosphate liberated was determined by measuring absorbance at 660 nm. A phosphate standard curve (0.5-10 nmol) was utilized for quantitation. Protoplasts transformed with pMON47203 liberated 3-fold more phosphate from sedoheptulose-1,7-bisphosphate than control protoplasts (Figure 9).

25 The high level of activity observed for the protoplasts transformed with wheat SBPase gene provides evidence that the SBPase protein detected by Western blot analysis is functional.

#### EXAMPLE 5

##### *Wheat SBPase expression in tobacco*

In order to test the effects of over-expressing SBPase from wheat in tobacco, pMON47200 (Fig. 5) was prepared and used as the transformation vector for tobacco plant  
30 transformation. Tobacco plant cells were transformed by Agrobacterium mediated

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transformation according to Horsch et al. (1985). The plant transformation vectors were mobilized into the ABI *Agrobacterium* strain by electroporation.

Growth chamber-grown tobacco transformant lines were generated and first screened by Western blot analysis to identify expressors using goat antibody raised against wheat-expressed SBPase. It was not, however, possible to separate the endogenous tobacco SBPase from the transgenic wheat SBPase using gradient SDS-PAGE gels or isoelectric focusing gels for these tobacco transformant lines.

#### EXAMPLE 6

##### *cDNA cloning of SBPase from Chlorella sorokiniana for expression in plants*

To determine the sequence of the gene encoding SBPase protein from *Chlorella sorokiniana*, several RT-PCR reactions were performed. One microgram of *Chlorella sorokiniana* RNA was combined with 100 pmol of oligo dT primer (Promega, Madison, WI), heated for 5 minutes at 75°C, and chilled on ice. First strand cDNA synthesis was performed using Superscript II<sup>TM</sup> reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) according to the manufacturer's protocol. The terminated reverse transcription reaction was diluted 1:7. Twenty microliters of the diluted first strand synthesis products were combined with 100 µM of each dNTP, 50 pmol of a degenerate primer with homology to the 5' end of the gene (5'- GGIACIATHTTYGGIGTITGG -3', SEQ ID NO: 10), 50 pmol of a gene specific primer with homology to the 3' end of the gene (5'- RTAICKIARIGTRTAYTTYTC -3', SEQ ID NO:11), and 5 Units of Taq polymerase (BRL/Life Technologies Inc, Gaithersburg, MD) in 100 µL. PCR cycling conditions were as follows: 95°C, 5 minutes (1 cycle) followed by 95°C, 40 seconds; 35°C, 1 minute; 72°C, 1 minute (5 cycles) followed by 95°C, 40 seconds; 40°C, 1 minute; 72°C, 1 minute (30 cycles). The 550 bp PCR product that was 250 bp larger than the expected size was gel purified, cloned into the PCR-Blunt cloning vector (Invitrogen, Carlsbad, CA), and transformed into competent *E. coli* cells. Plasmid was purified from clones containing insert that had been selected on media containing kanamycin. Sequence analysis revealed that the predicted amino acid sequence of this 550 bp PCR product is 41% identical to the *Chlamydomonas reinhardtii* sequence. Residues 226-251 of the *Chlamydomonas reinhardtii* sequence was 81% identical to the *Chlorella* sequence and residues 252-287 of the *Chlamydomonas* sequence was 86% identical but there was a gap encoding 62 *Chlorella*, residues presumably corresponding to an intron. Therefore this fragment was most likely

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amplified from contaminating genomic DNA. The 297 bp of coding sequence was used to design primers for 5' RACE and 3' RACE to identify the sequence of the rest of the gene.

To clone the remaining 5' sequence of the SBPase, a modified anchored PCR procedure for the rapid amplification of cDNA ends (Frohman, 1990; Jain et al., 1992) was used. Nine hundred nanograms of total *Chlorella sorokiniana* RNA was combined with 20 pmol of a gene-specific primer (5'-GATGGTCTCGGTCTCCTTACG-3', SEQ ID NO:13), heated to 75°C for 5 minutes, and chilled on ice. First strand DNA synthesis was performed using Thermoscript™ reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) at 55°C according to the supplier's protocol. The terminated reverse transcription reaction was treated with one unit of ribonuclease H for 20 minutes at 37°C, 5 minutes at 95°C, and chilled on ice. Excess primers and dNTPs were removed by centrifugation at 2,000 x g through an Ultrafree-MC filterfuge (30,000 MW cutoff, Millipore, Bedford, MA), and the retentate was concentrated to 15 µL on a Savant Speedvac (Savant Instruments, Holbrook, NY). The first-strand synthesis products were combined with 10 µL of tailing mix (1X tailing buffer [BRL/Life Technologies Inc, Gaithersburg, MD], 0.4 mM dATP, 10 units of terminal deoxytransferase) and incubated at 37°C for 10 minutes. The reaction mixture was heated to 95°C for 5 minutes, diluted to 0.2 mL with 10 mM Tris, pH 8.5, and utilized as a cDNA pool. A mixture of 20 µL of the cDNA pool, 10 µL PWO™ polymerase 10X buffer (BRL/Life Technologies Inc, Gaithersburg, MD), 100 µM of each dNTP, 25 pmol of a gene specific primer (SEQ ID NO:13), 10 pmol of the poly(dT) adaptor primer (5'-GGGTCGACATTCTAGACAGAATTCGTGGATCC(T)<sub>21</sub>-3'; SEQ ID NO:5), and 5 units of PWO™ polymerase (BRL/Life Technologies Inc, Gaithersburg, MD) in 100 µL was amplified. PCR cycling conditions were as follows: 95°C, 2 minutes; 45°C, 5 minutes; 72°C, 40 minutes (1 cycle) followed by 95°C, 50 seconds; 48°C, 1 minute; 72°C, 1 minute (3 cycles). The PCR products were purified away from the excess primers by ethanol precipitation. The pellet was resuspended in 50 µL of water and combined with 10 µL of PWO™ polymerase 10X buffer (BRL/Life Technologies Inc, Gaithersburg, MD) in 100µL, 50 µM of each dNTP, 50 pmol a new nested gene specific primer (5'- CAGCCACTTGCCATCGTC -3', SEQ ID NO:14), 50 pmol of an adaptor primer (5'-GGGTCGACATTCTAGACAGAA-3', SEQ ID NO:7), 5 Units of PWO™ polymerase (BRL/Life Technologies Inc, Gaithersburg, MD). PCR cycling conditions were as follows: 95°C, 40 seconds; 48°C, 1 minute; 72°C, 1 minute, 30 seconds (30 cycles). The



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400 bp PCR product was gel purified, subcloned into the PCR-Blunt TOPO II cloning vector (Invitrogen, Carlsbad, CA) and transformed into competent *E. coli* cells for further characterization. Sequence analysis showed that this PCR fragment was part of the 5' sequence of the *Chlorella sorokiniana* SBPase.

5 This sequence was then used to design additional primers to clone the remaining 5' sequence of the SBPase using the modified anchored PCR procedure for the rapid amplification of cDNA ends (Frohman, 1990; Jain et al., 1992). Nine hundred nanograms of total RNA was combined with 20 pmol of a gene-specific primer (5'-GATGGTCTCGGTCTCCTTCACG-3', SEQ ID NO:15), heated to 75°C for 5 minutes, and chilled on ice. First strand DNA synthesis  
10 was performed using Thermoscript<sup>TM</sup> reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) at 55°C according to the supplier's protocol. The terminated reverse transcription reaction was treated with one unit of ribonuclease H for 20 minutes at 37°C, 5 minutes at 95°C, and chilled on ice. Excess primers and dNTPs were removed by centrifugation at 2,000 x g through an Ultrafree-MC filterfuge (30,000 MW cutoff, Millipore, Bedford, MA),  
15 and the retentate was concentrated to 15 µL on a Savant Speedvac (Savant Instruments, Holbrook, NY). The first-strand synthesis products were combined with 10 µL of tailing mix (1X tailing buffer [BRL/Life Technologies Inc, Gaithersburg, MD], 0.4 mM dATP, 10 units of terminal deoxytransferase) and incubated at 37°C for 10 minutes. The reaction mixture was heated to 95°C for 5 minutes, diluted to 0.2 mL with TE, pH 8.0, and utilized as a cDNA pool. A  
20 mixture of 20 µL of the cDNA pool, 10 µL HotStarTaq<sup>TM</sup> polymerase 10X buffer (Qiagen, Valencia, CA), 20 µL of Q 5X buffer (Qiagen, Valencia, CA) 50 µM of each dNTP, 25 pmol of a gene specific primer (5'-TCCTCAGAGCACGCCAGCTTGC-3', SEQ ID NO:16), 10 pmol of the poly(dT) adaptor primer (5'-GGGTCGACATTCTAGACAGAATTTCGTGGATCC(T)<sub>21</sub>-3'; SEQ ID NO:5), and 5 units of HotStarTaq<sup>TM</sup> polymerase (Qiagen, Valencia, CA) in 100 µL was  
25 amplified. PCR cycling conditions were as follows: 95°C, 15 minutes; 45°C, 5 minutes; 72°C, 40 minutes (1 cycle) followed by 95°C, 50 seconds; 48°C, 1 minute; 72°C, 1 minute (3 cycles). The PCR products were purified away from the excess primers by ethanol precipitation. The pellet was resuspended in 50 µL of water and combined with 10 µL HotStarTaq<sup>TM</sup> polymerase 10X buffer (Qiagen, Valencia, CA), 20 µL of Q 5X buffer (Qiagen, Valencia, CA), 50 µM of  
30 each dNTP 50 pmol of new nested gene specific primer (5'-

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AGCTGCTCATCGCCGAACGAGTTG-3'. SEQ ID NO:17), 50 pmol of an adaptor primer (5'-GGGTCGACATTCTAGACAGAA-3', SEQ ID NO:7), and 5 Units of HotStarTaq<sup>TM</sup> polymerase (Qiagen, Valencia, CA). PCR cycling conditions were as follows: 95°C, 15 minutes (1 cycle) followed by 95°C, 40 seconds; 50°C, 1 minute; 72°C, 1 minute, 30 seconds (30 cycles).

5 The 380 bp PCR product was gel purified, subcloned into the PCR-Blunt TOPO II cloning vector (Invitrogen, Carlsbad, CA) and transformed into competent *E. coli* cells for further characterization. Sequence analysis showed that this PCR fragment contains the remaining 5' sequence of the *Chlorella* SBPase.

PCR was used to isolate the remaining 3' sequence of the SBPase from a *Chlorella*  
 10 *sorokiniana* cDNA library in pSport1 constructed using Superscript<sup>TM</sup> Plasmid System for cDNA Synthesis and Plasmid Cloning (BRL/Life Technologies Inc, Gaithersburg, MD) according to the supplier's protocols. One microgram of *Chlorella sorokiniana* RNA was combined with 100 pmol of oligo dT primer (Promega, Madison, WI), heated for 5 minutes at 75°C, and chilled on ice. First strand cDNA synthesis was performed using Thermoscript<sup>TM</sup>  
 15 reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) at 60°C according to the manufacturer's protocol instead of the Superscript<sup>TM</sup> reverse transcriptase (BRL/Life Technologies Inc, Gaithersburg, MD) provided with the kit. A mixture of 200 ng of library DNA, 10 µL HotStarTaq<sup>TM</sup> polymerase 10X buffer (Qiagen, Valencia, CA), 50 µM of each dNTP, 50 pmol of a gene specific primer (5'- AGTTCCTGCTGCAGGACGATGG -3'; SEQ ID NO:18), 50 pmol of the vector specific primer (5'-CCCAGTCACGACGTTGTAAAACG -3';  
 20 SEQ ID NO:19), and 5 units of HotStarTaq<sup>TM</sup> polymerase (Qiagen, Valencia, CA) in 100 µL was amplified. PCR cycling conditions were as follows: 95°C, 15 minutes (1 cycle) followed by 95°C, 40 seconds; 62°C, 1 minute; 72°C, 1 minute, 30 seconds (30 cycles). The overlapping 5' and 3' sequences were assembled to give the full length *Chlorella sorokiniana* SBPase  
 25 sequence (SEQ ID NO:20) with predicted amino acid sequence (SEQ ID NO:12).

Table 1. Comparison of *Chlorella sorokiniana* SBPase derived amino acid sequence (SEQ ID NO:12) with known SBPase amino acid sequences. The alignments were made using the Bestfit program to determine percentage homology in the table below. BestFit program uses the *local*  
 30 *homology* algorithm of Smith and Waterman (1981) to find the best segment of similarity between two sequences.

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	Similarity	Identity
<i>Arabidopsis thaliana</i>	76.7%	71.6%
<i>Chlamydomonas reinhardtii</i>	82.0%	78.0%
<i>Spinacia oleracea</i>	74.3%	68.4%
<i>Triticum aestivum</i>	72.0%	66.8%

**EXAMPLE 7***Cloning genes encoding dual FBPase and SBPase activity*

Bisphosphatase genes from the facultative chemoautotroph *Ralstonia eutropha* (*Alcaligenes eutrophus*) and from the algae *Synechococcus lepoliensis* encode proteins with a dual activity, FBPase and SBPase, in the Calvin cycle (Yoo and Bowien, 1995; Gerbling et al., 1986). A gene encoding a protein with SBPase activity will be cloned from *Ralstonia eutropha* using PCR based on the sequence described in Yoo and Bowien(1995). This gene will then be fused to a chloroplast transit peptide, in order to target the SBPase protein to the plastid.

**EXAMPLE 8***Chlorella and Ralstonia SBPase expression in tobacco*

In order to test the effects of over-expressing wild type or deregulated *Chlorella* as well as *Ralstonia* SBPase in tobacco, each gene will first be cloned into the pCR<sup>®</sup>-Blunt II<sup>™</sup> cloning vector (Invitrogen, Carlsbad, CA). Each vector will then be digested with EcoRI and the gel purified fragments encoding each of the SBPases will be ligated individually to EcoRI-linerarized pMON10098 (Figure 4). Each of the 3 vectors will be used for tobacco plant transformation, as described in Horsch et al. (1985). The plant transformation vectors are mobilized into the ABI *Agrobacterium* strain by electroporation.

Growth chamber-grown tobacco transformant lines are generated and first screened by Western blot analysis to identify expressors using goat antibody raised against wheat-expressed SBPase. Subsequently, for wild type *Chlorella*-SBPase, deregulated *Chlorella* SBPase- and *Ralstonia* SBPase-expressing tobacco lines, leaf samples taken at various stages of plant development will be analyzed for diurnal changes in leaf nonstructural carbohydrates (sucrose, glucose, and hydrolyzed starch into glucose) by means of the Sucrose/D-Glucose/D-Fructose kit (Boehringer Mannheim, Germany).

Thirty to fifty milligrams of frozen tobacco leaf tissue samples will be incubated in 1 mL of 85°C water for 15 minutes. Tubes will be centrifuged for 1 minute at 10,000 x g and the supernatants saved for soluble sugar analysis. The pellet will be resuspended in 1 mL of 85°C

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water, mixed with a Vortex, and centrifuged as described above. The supernatant will be carefully removed and added to the previous supernatant fraction for soluble sugar (sucrose and glucose) analysis by the Sucrose/D-Glucose/D-Fructose kit (Boehringer Mannheim, Germany).

The starch will be extracted from the pellet using by adding 3 mL of 0.1 M sodium acetate, pH 5.6 to the pellet and incubating at 90°C for 10 minutes. Once cool, 3 mL of 1% amyloglucosidase in 0.1 M sodium acetate, pH 5.6 will be added and vortexed. The samples will be incubated in a water bath at 50°C for 3 hours, vortexing every half hour. After centrifugation in a table top centrifuge at 900 x g for 30 minutes, the supernatants will be analyzed for glucose content. The free glucose will be adjusted to anhydrous glucose (as it occurs in starch by multiplying by the ratio 162/182).

Alteration of carbon assimilation in the transgene plant tissue can be monitored by metabolic profiling using standard HPLC, mass spectroscopy, and enzymatic based metabolite assays.

#### EXAMPLE 9

##### 15 *Deregulation of SBPase*

In the dark, wheat SBPase is inactive due to disulfide bond formation between Cys-52 and Cys-57 (numbers correspond to mature wheat SBPase, CA Raines et al. 1999. *J. Exp. Bot.* 50: 1-8). In the light, thioredoxin reduces the disulfide linkage resulting in an active enzyme. Modifying the Cys residues through mutagenesis would prevent formation of the disulfide bond and therefore prevent inactivation of the protein by oxidation. Site-directed mutagenesis to change Cys-110 and Cys-115 (numbers correspond to *Chlorella* SBPase including the CTP, SEQ ID NO:12) to Ser will be performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using a mutagenic primer (5'-AAGGTGCGCACCGCCTCGTCCGGCGGCACCGCCTCCGTCAACTCGTTCGGCGATG - 25 3'; SEQ ID NO: 21) according to the manufacturer's protocol. The resulting sequence SEQ ID NO:22 will be inserted into the appropriate transformation vector and will be used to transform tobacco and corn. The resulting plants will be tested for expression of the SBPase in the plant tissues and enzymatic assays will be run to confirm activity. The plants will be analyzed to determine the improved assimilation of carbon in the plant and its export and storage to sink 30 tissues. The predicted amino acid sequence resulted from the DNA sequence (SEQ ID NO:22) is shown as SEQ ID NO:23.

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It should also be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

5 All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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- 30 -

## CLAIMS:

1. A method for improving the assimilation of carbon in a plant comprising the steps of:
  - 5 a). inserting into the genome of a plant a nucleic acid sequence comprising in the 5' to 3' direction,
    - i). a promoter that functions in the cells of said plant, said promoter operably linked to;
    - 10 ii). a structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme, said structural nucleic acid sequence operably linked to;
    - iii). a 3' non-translated nucleic acid sequence that functions in said cells of said plant to cause transcriptional termination;
  - b). obtaining transformed plant cells containing the nucleic acid sequence of step (a);
  - 15 and
  - c). regenerating from said transformed plant cells a transformed plant that overexpresses said sedoheptulose 1,7-bisphosphatase enzyme in said plant cells.
2. The method of claim 1 wherein said structural nucleic acid sequence that causes the  
20 production of a sedoheptulose 1,7-bisphosphatase enzyme is from a source heterologous with respect to the plant into which the structural nucleic acid sequence is inserted.
3. The method of claim 2 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from a bacterial microorganism.
4. The method of claim 3 wherein said structural nucleic acid sequence that causes the  
25 production of a sedoheptulose 1,7-bisphosphatase enzyme is from *Ralstonia*.
5. The method of claim 2 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from a green algae.
6. The method of claim 5 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from *Chlorella*.
- 30 7. The method of claim 2 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from a plant species heterologous with respect to the plant into which the structural nucleic acid sequence is inserted.



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8. The method of claim 1 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is endogenous to said plant into which it is inserted and said promoter operably linked thereto is capable of expressing said inserted, endogenous sedoheptulose 1,7-bisphosphatase enzyme at a level greater than that of a plant not transformed to contain said inserted structural nucleic acid sequence.
9. The method of claim 1 wherein said plant is a monocotyledonous plant.
10. The method of claim 1 wherein said plant is a dicotyledonous plant.
11. The method of claim 1 wherein said plant is selected from the group consisting of maize, wheat, rice, potato alfalfa, barley, cotton, soybean, canola, sunflower, and sugarbeet.
12. The method of claim 1 wherein said sedoheptulose 1,7-bisphosphatase enzyme is deregulated with respect to light.
13. An isolated nucleic acid sequence comprising:
- (a) a promoter that functions in the cells of a plant, said promoter operably linked to;
  - (b) a structural nucleic acid sequence in sense orientation that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme, said structural nucleic acid sequence operably linked to, and
  - (c) a 3' non-translated nucleic acid sequence that functions in said cells of said plant to cause transcriptional termination.
14. The isolated nucleic acid sequence of claim 13 further comprising an intron.
15. The isolated nucleic acid sequence of claim 13 further comprising a 5' untranslated leader sequence.
16. The isolated nucleic acid sequence of claim 13 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from a bacterial microorganism.
17. The isolated nucleic acid sequence of claim 16 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from *Ralstonia*.
18. The isolated nucleic acid sequence of claim 13 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from a green algae.

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19. The isolated nucleic acid sequence of claim 18 wherein said structural nucleic acid sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from *Chlorella*.
20. The isolated nucleic acid sequence of claim 19 wherein said structural nucleic acid  
5 sequence that causes the production of a sedoheptulose 1,7-bisphosphatase enzyme is from a plant.
21. The isolated nucleic acid sequence of claim 13 wherein said sedoheptulose 1,7-bisphosphatase enzyme is deregulated with respect to light.
22. An isolated nucleic acid sequence comprising the nucleotide sequence of SEQ ID NO:20,  
10 or a degenerate variant of SEQ ID NO:20.
23. An isolated nucleic acid sequence comprising a sequence that encodes a polypeptide with the amino acid sequence of SEQ ID NO:12 or SEQ ID NO:12 with conservative amino acid substitutions.
24. An isolated nucleic acid sequence comprising a sequence at least 85% identical to SEQ  
15 ID NO:20.
25. An isolated nucleic acid sequence comprising a sequence that encodes a polypeptide the amino acid sequence of which is at least 85% similar to SEQ ID NO:12.

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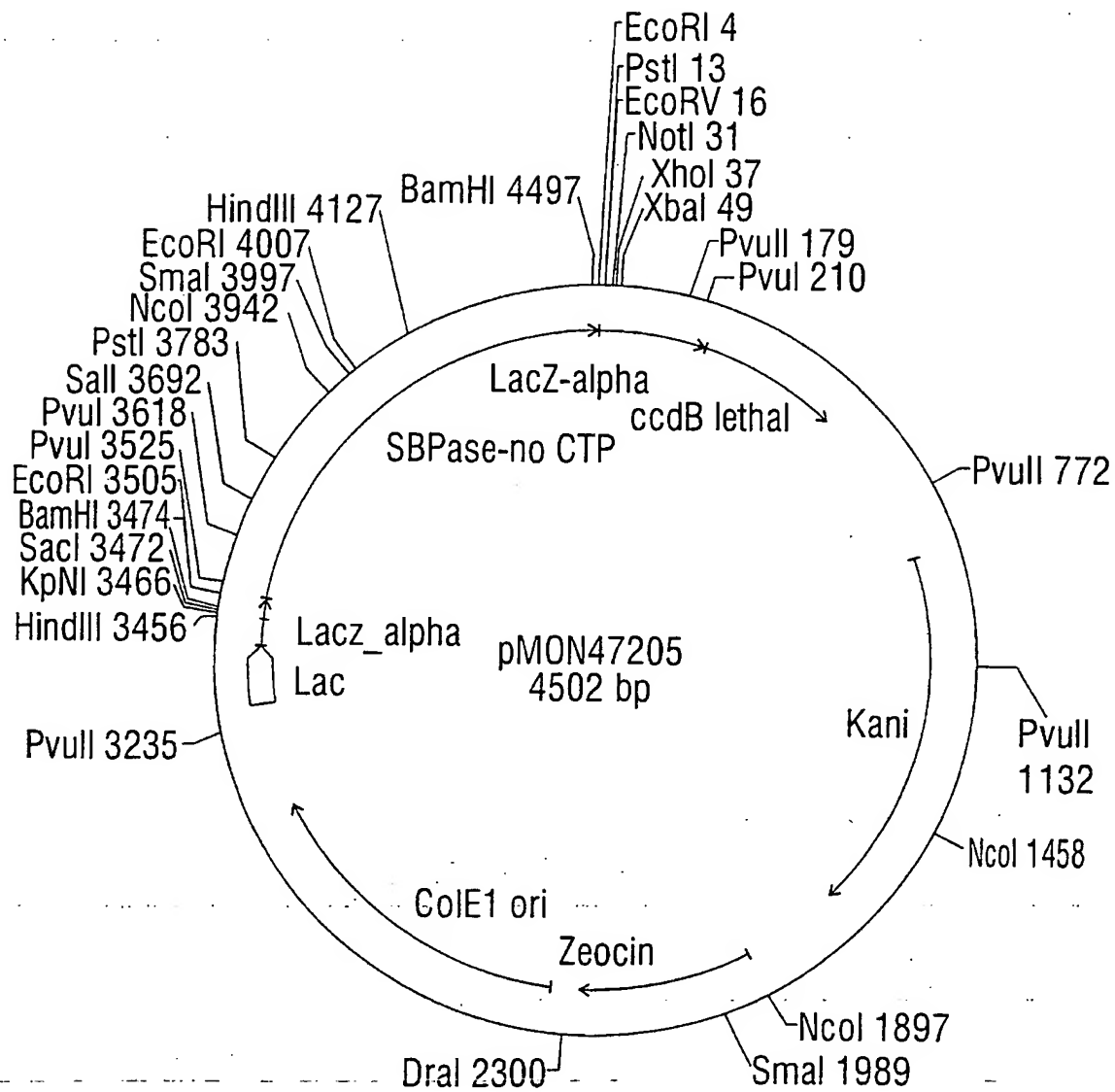


FIG. 1

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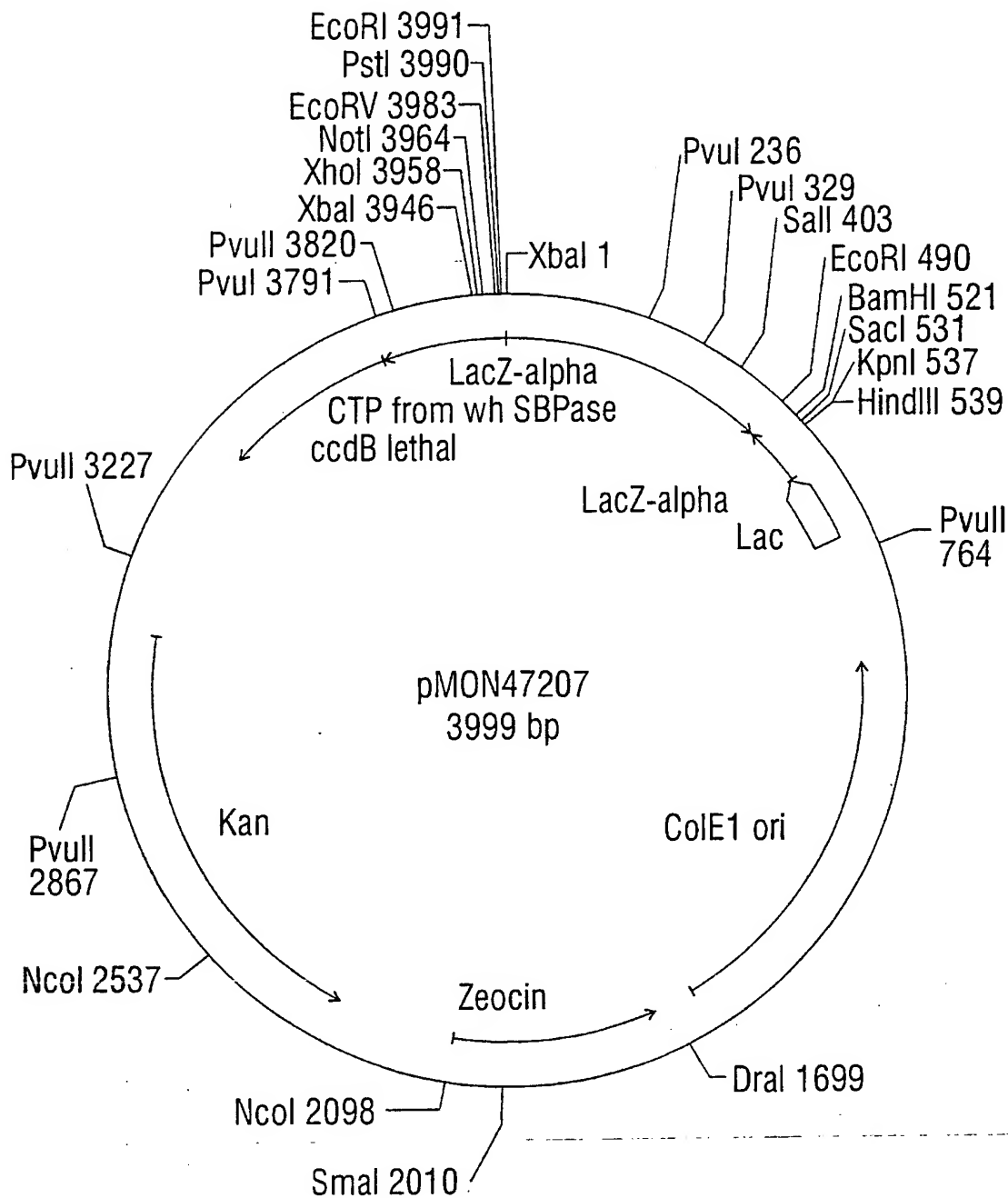


FIG. 2

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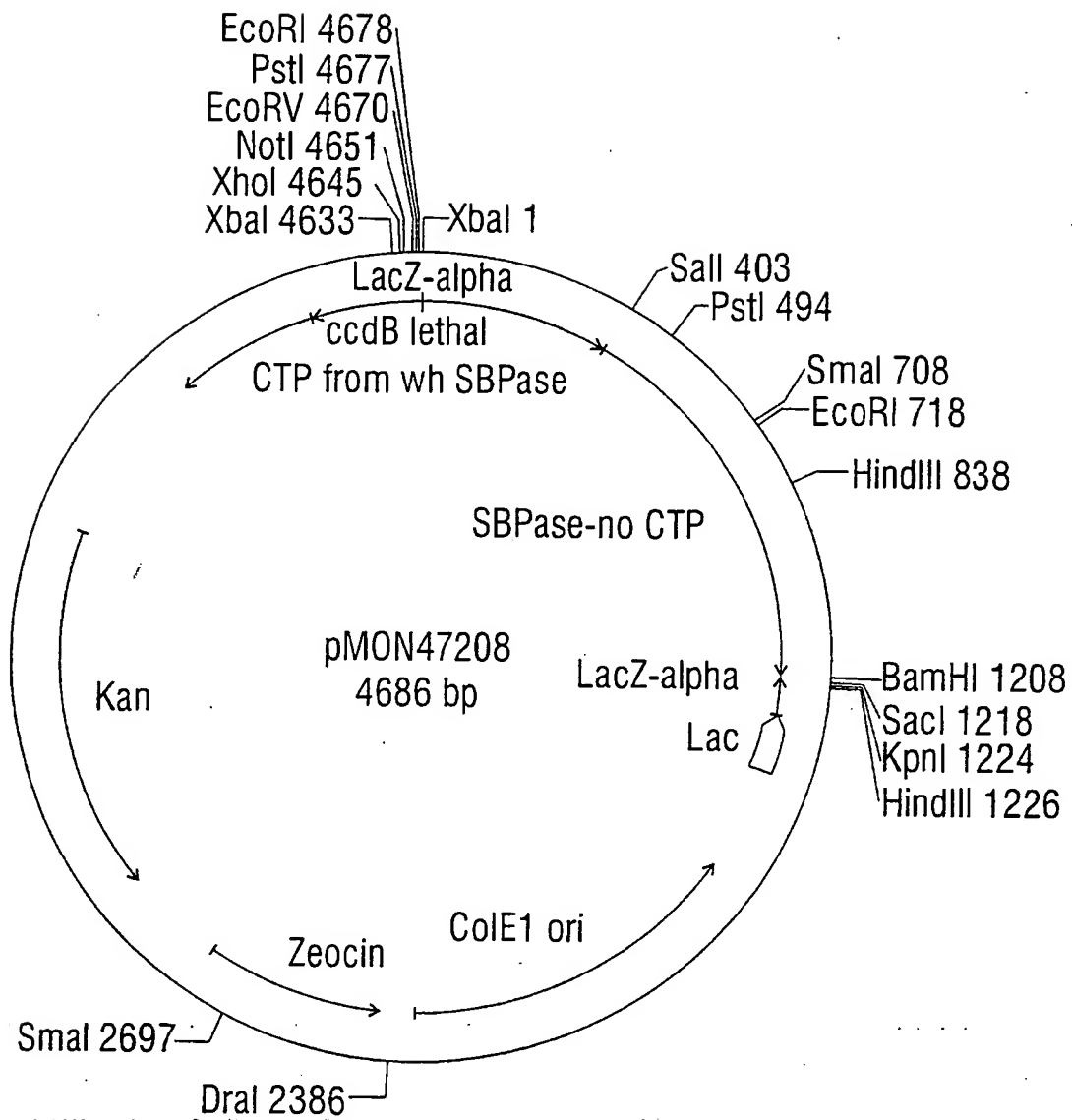


FIG. 3

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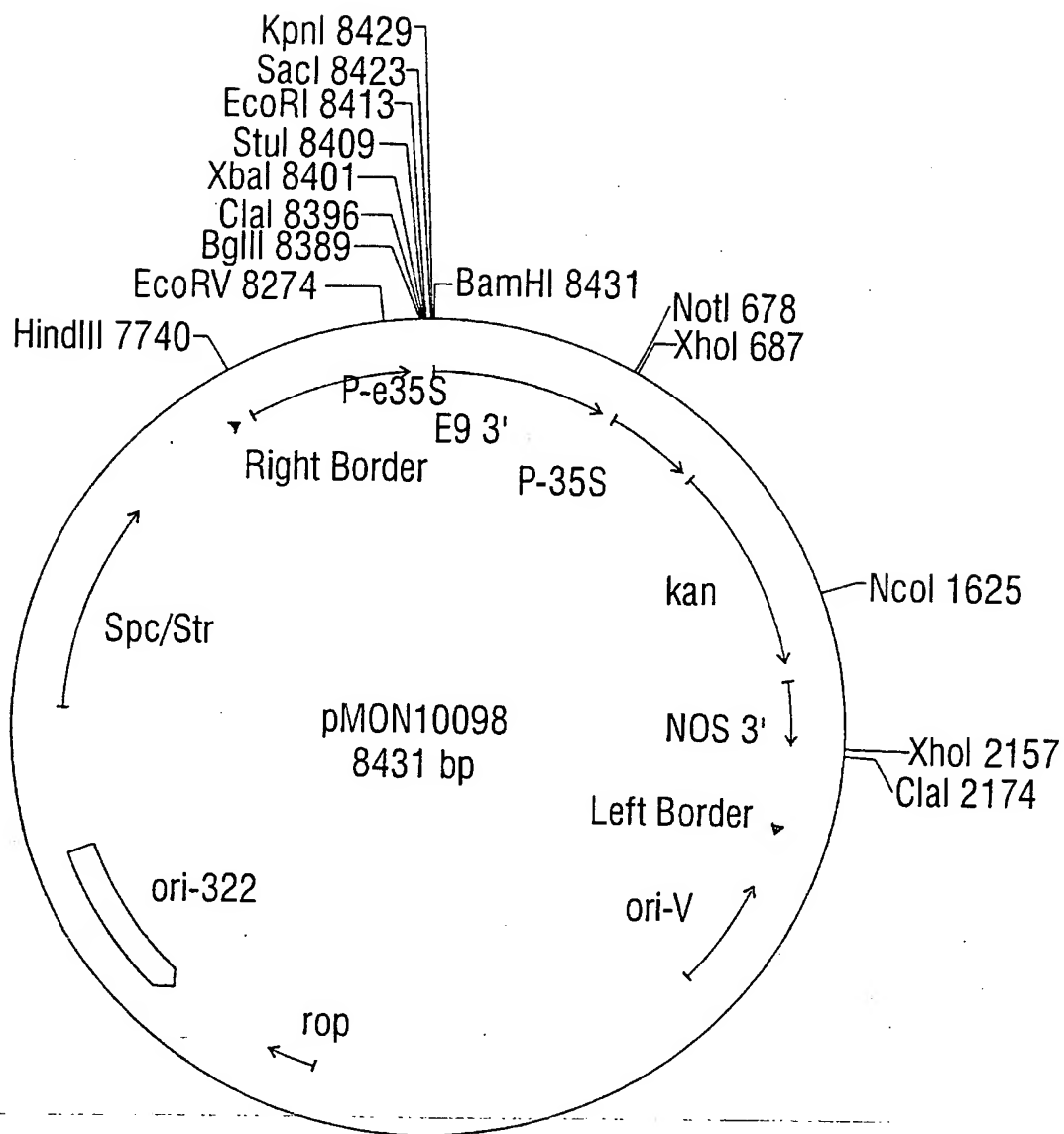


FIG. 4

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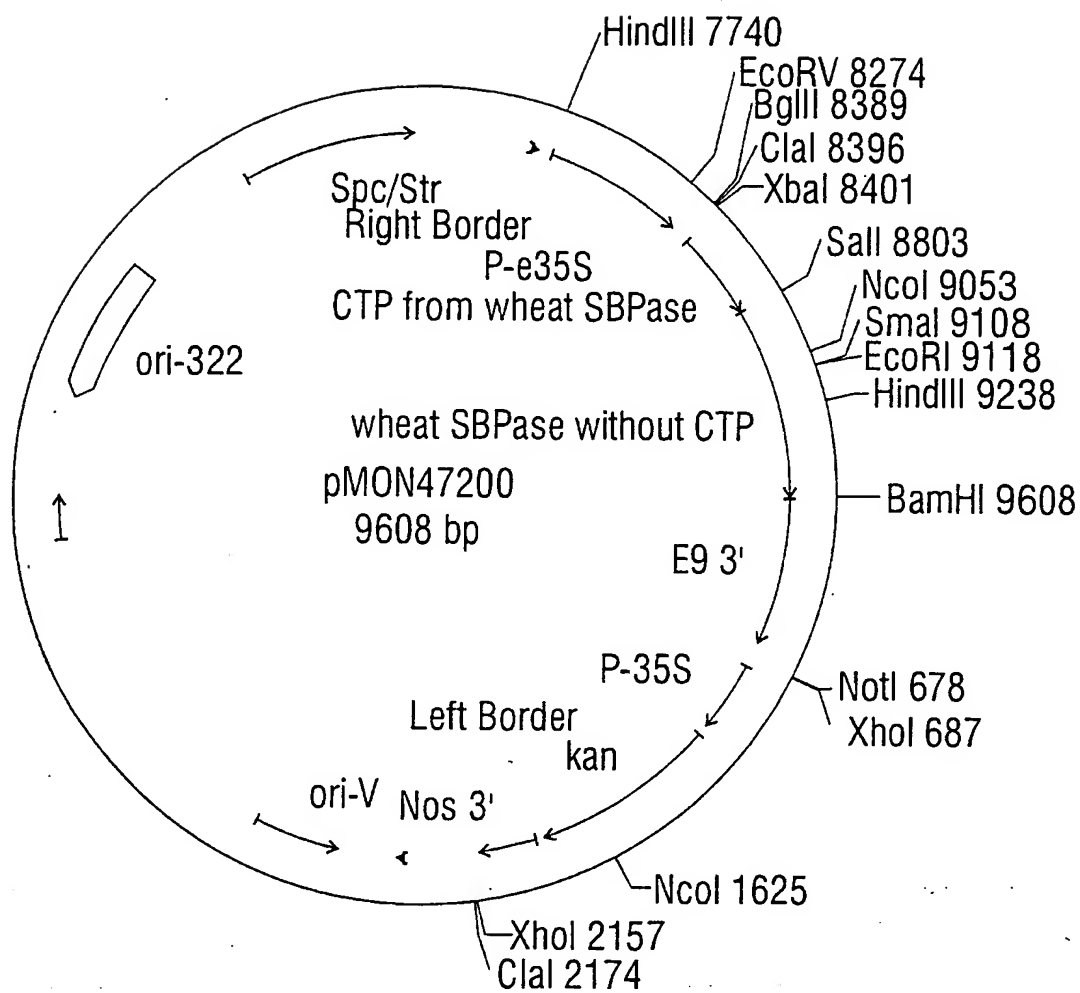


FIG. 5

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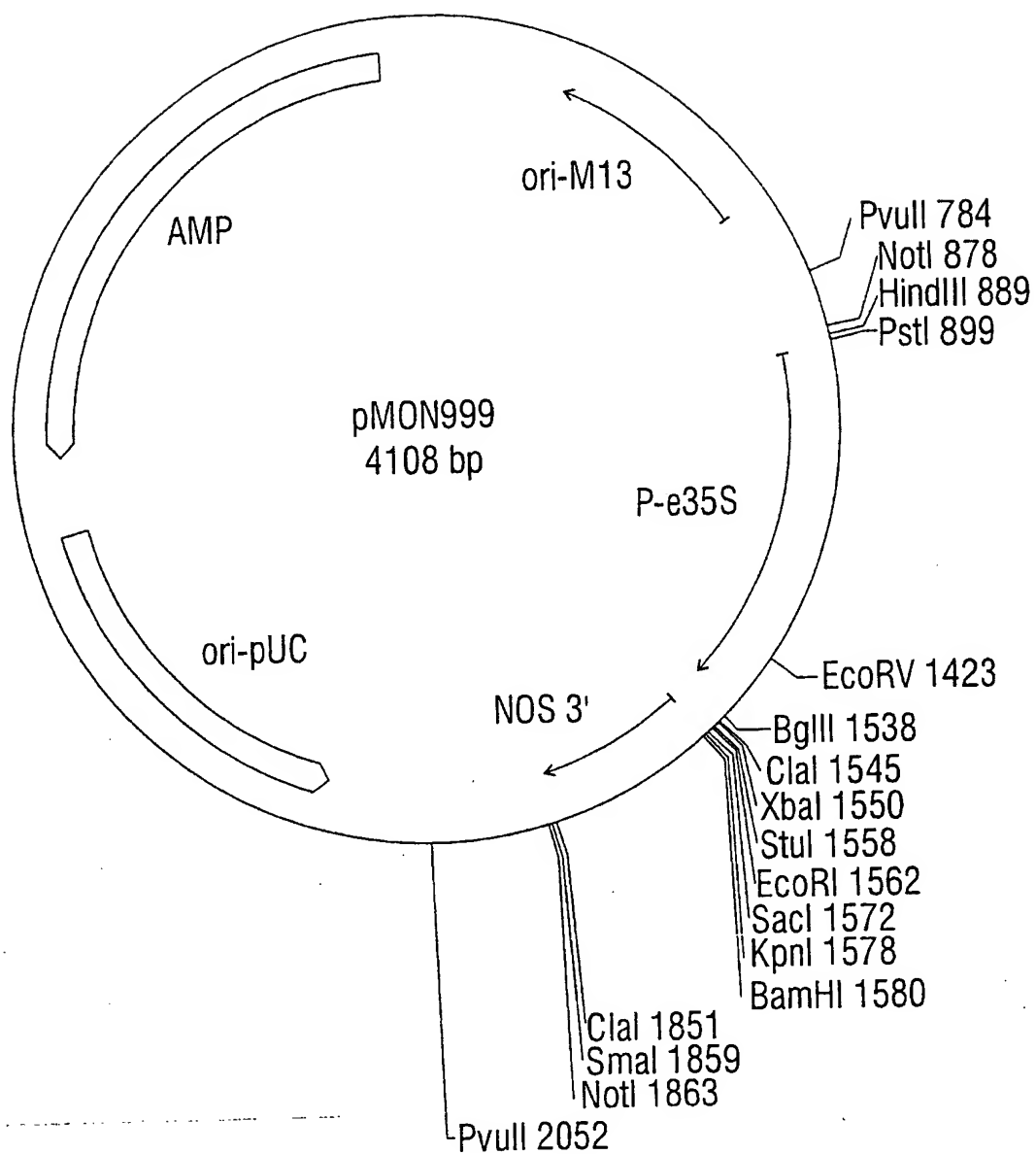


FIG. 6



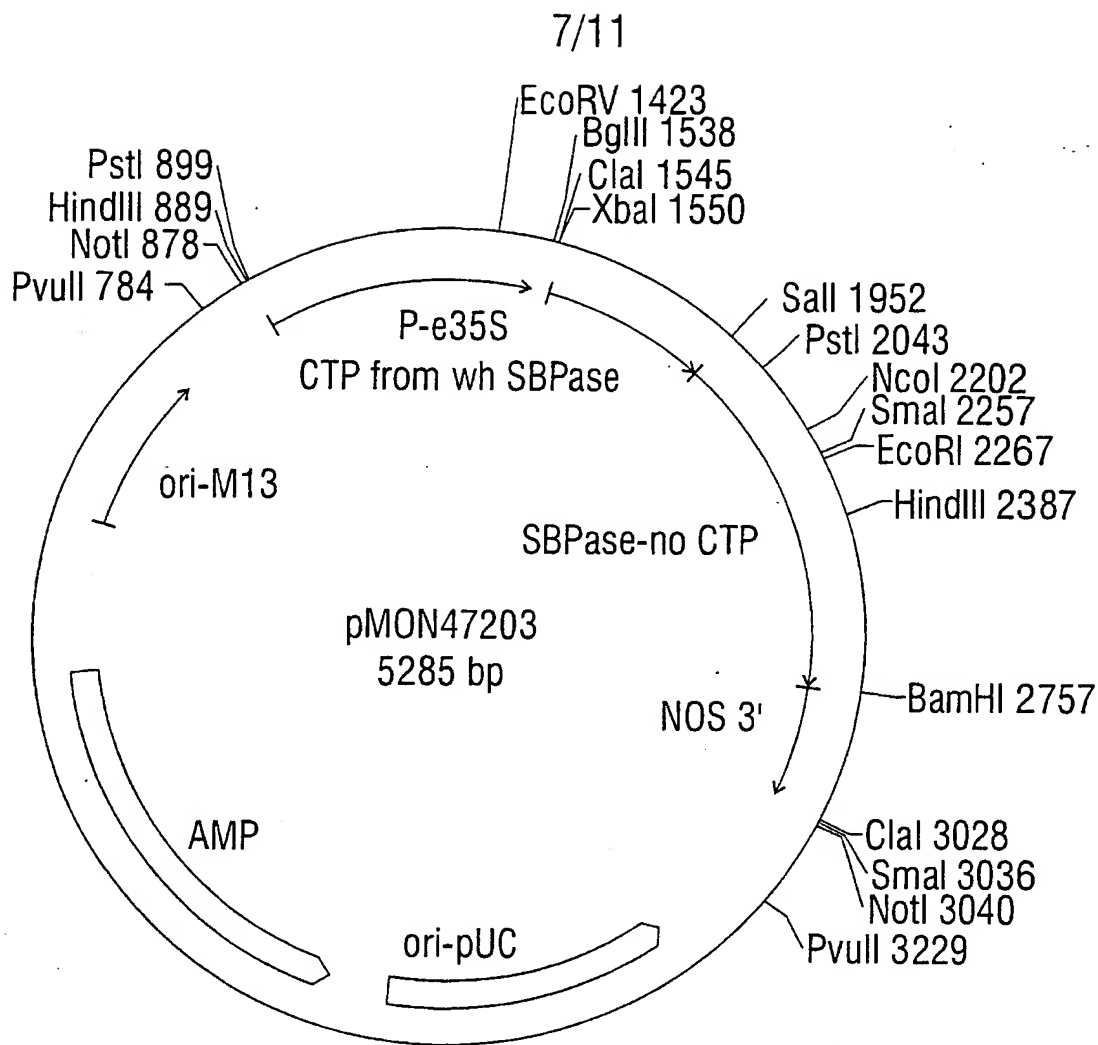


FIG. 7

Control protoplasts  
SBPase protoplasts  
Wheat leaf

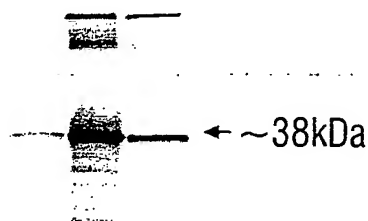


FIG. 8

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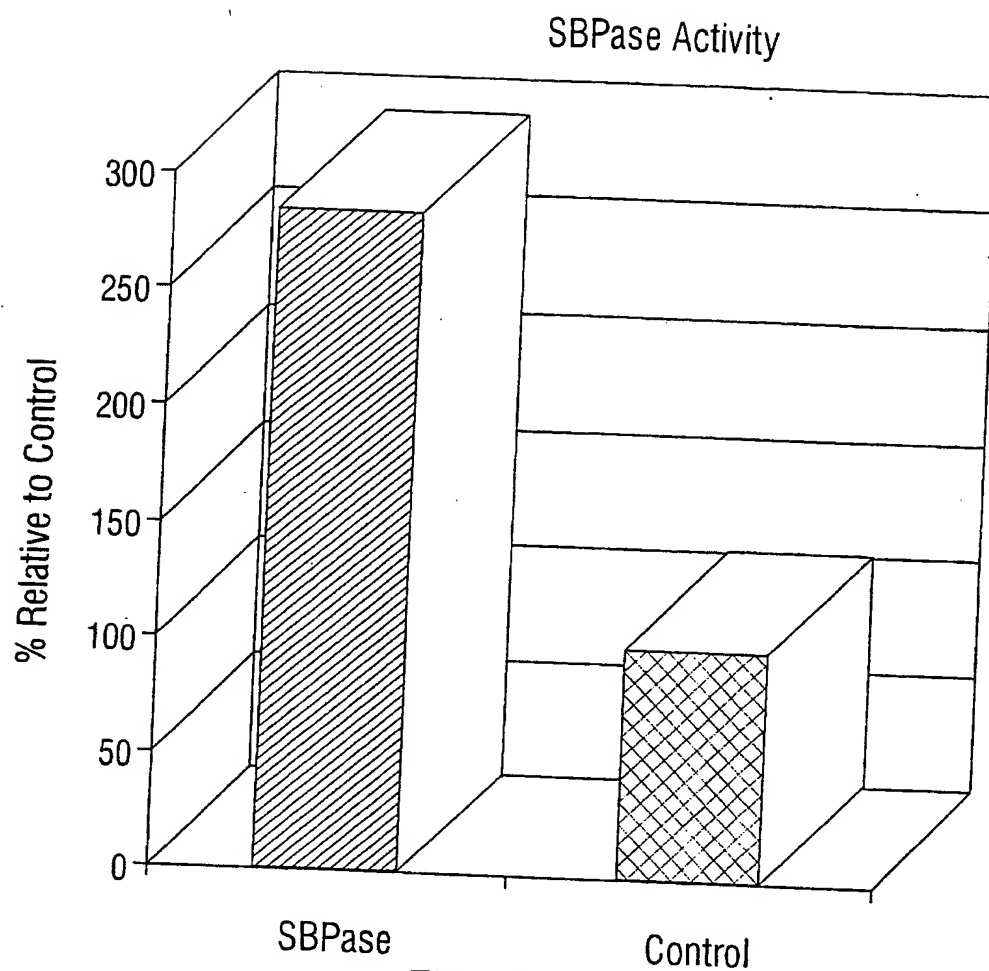


FIG. 9

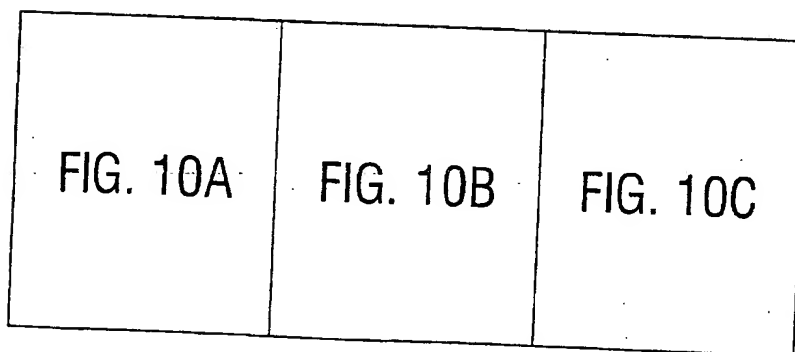


FIG. 10

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1      50
S17P_CHLSA      ~~~~~MQATAVAT AAPAARVATT GKAATGVKAA PRVAVRAAGA
S17P_CHLRE      ~~~~~MAAMMM RQKVAGAIAG ERRSAVAPKM GRAAT...A P.VVASANA
S17P_SPIOL      MET.SMACCS RSIVLPRVSP QHSSALV..P S...SINLKS LK.SSSLFGE
S17P_ARATH      MET.SIACYS RGILPPSVSS QRSSTLVSP SYSTSSSFKR LK.SSSIFGD
S17P_WHEAT      METVAAAGYA HG.....AA TRSPACCAAM SFSQSYRPKA ARPATSFYGE

51      100
S17P_CHLSA      SASSSFATGA RL..SAKASR TAARRAAVAA QAKIGDTLEE FLLEATPDPK
S17P_CHLRE      SAFKGAAVTA RVKASTRAAR VQSRRTAVLT QAKIGDSLAE FLVEATPDPK
S17P_SPIOL      SLRM.TTKSS ..V...RVNK AKN..SSLVT KCELGDSLEE FLAKATTDKG
S17P_ARATH      SLRL.APKSQ ..L...KATK AKSNGASTVT KCEIGQSLEE FLAQATPDKG
S17P_WHEAT      SLRANTARTS ..FPAGRQSK AASR.AALT RCALGDSLEE FLTKATPDKN

101      150
S17P_CHLSA      LRQLMMSMSE AIRTIAYKVR TASCGGTACV NSFGDEQLAV DLLADKLLFE
S17P_CHLRE      LRHVMMMSMAE ATRTIAHKVR TASCAGTACV NSFGDEQLAV DMVADKLLFE
S17P_SPIOL      LIRLMMCMGE ALRTIGFKVR TASCGGTQCV NTFGDEQLAI DVLADKLLFE
S17P_ARATH      LRTLLMCMGE ALRTIAFKVR TASCGGTACV NSFGDEQLAV DMLADKLLFE
S17P_WHEAT      LIRLLICMGE AMRTIAFKVR TASCGGTACV NSFGDEQLAV DMLADKLLFE

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FIG. 10A

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151	S17P_CHLSA	200	ALYSGGCKL	ACSEEVPEPL	DLGGE...GF	SVAFDPLDGS	SIVDTNFSVG
	S17P_CHLRE		ALKYSHVCKL	ACSEEVPEPV	DMGGE...GF	CVAFDPLDGS	SSSDTNFAVG
	S17P_SPIOL		ALNYSHFCKY	ACSEELPELQ	DMGGPVDGGF	SVAFDPLDGS	SIVDTNFSVG
	S17P_ARATH		ALQYSHVCKY	ACSEEVPELQ	DMGGPVEGGF	SVAFDPLDGS	SIVDTNFTVG
	S17P_WHEAT		ALEYSHVCKY	ACSEEVPELQ	DMGGPVEGGF	SVAFDPLDGS	SIVDTNFTVG
201	S17P_CHLSA	250	TIFGVWPGDK	LTGITGRQQA	AAGMGIYGPR	TVFCIALKDA	PGCHEFLLQD
	S17P_CHLRE		TIFGVWPGDK	LTNITGREQV	AAGMGIYGPR	TVFCIALKDA	PGCHEFLLMD
	S17P_SPIOL		TIFGVWPGDK	LTGVTGRDQV	AAAMGIYGPR	TTYVLAKDY	PGTHEFLLLD
	S17P_ARATH		TIFGVWPGDK	LTGITGGDQV	AAAMGIYGPR	TTYVLAVKGF	PGTHEFLLLD
	S17P_WHEAT		TIFGVWPGDK	LTGVTGGDQV	AAAMGIYGPR	TTFVVALKDC	PGTHEFLLLD
251	S17P_CHLSA	300	DGKWLHVKET	ETIGEGKMFS	PGNLRATFDN	PAYEKLIAYY	IGEKYTLRYT
	S17P_CHLRE		DGKWMHVKET	THIGEGKMFA	PGNLRATFDN	PAYERLINFY	LGEKYTLRYT
	S17P_SPIOL		EGKWQHVKET	TEINEGKLCF	PGNLRATSDN	ADYAKLIQYY	IKEKYTLRYT
	S17P_ARATH		EGKWQHVKET	TEIAEGKMFS	PGNLRATFDN	SEYSKLIDYY	VKEKYTLRYT
	S17P_WHEAT		EGKWQHVKDT	TSIGEGKMFS	PGNLRATFDN	PDYDKLVNYY	VKEKYTLRYT

FIG. 10B

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301			350
S17P_CHLSA	GGMVPDVFI	IVKEKGVFTN	VISPSTKAKL RLLFEVAPLA LLVEKAGGAS
S17P_CHLRE	GGIVPDLFI	IVKEKGVFTN	LTSPSTKAKL RILFEVAPLA LLIEKAGGAS
S17P_SPIOL	GGMVPDVNQI	IVKEKGIFTN	VISPTAKAKL RLLFEVAPLG FLIEKAGGHS
S17P_ARATH	GGMVPDVNQI	IVKEKGIFTN	VTSPSTAKAKL RLLFEVAPLG LLIEKAGGFS
S17P_WHEAT	GGMVPDVNQI	IVKEKGIFTN	VTSPSTAKAKL RLLFEVAPLG FLIEKAGGHS
	351		400
S17P_CHLSA	SCDGLCVSGL	DVEVKQHDQR	TQICYGSKGE VRRFEEYMYG NSPRFSEVTA
S17P_CHLRE	SCDGKAVSAL	DIPILVCDQR	TQICYGSIGE VRRFEEYMYG TSPRFSEKVV
S17P_SPIOL	S.EGT.KSVL	DIEVKNLDDR	TQVAYGSLNE IIRFEKTLYG SS.RLEEPVP
S17P_ARATH	S.DGH.KSVL	DKTIINLDDR	TQVAYGSKNE IIRFEETLYG TS.RLKN.VP
S17P_WHEAT	S.DGK.QSVL	DKVISVLDER	TQVAYGSKNE IIRFEETLYG SS.RLAASAT
	401		
S17P_CHLSA	~~~~~		
S17P_CHLRE	A~~~~		
S17P_SPIOL	VGAAA		
S17P_ARATH	IGVTA		

FIG. 10C

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## SEQUENCE LISTING

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 Staub, Robin L

<120> Expression of Sedoheptulose 1,7 Bisphosphatase in  
 Transgenic Plants

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agtgtggcgt tcgacccccct tgacgggtcc agcatcgtgg acaccaactt caccgtggga 360
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cctgattatg acaagcttgt caactactat gtgaaggaga agtacactct gcgttacacc 660
ggaggaatgg tccctgatgt caaccagatc atcgtgaagg agaaggcat cttcacgaac 720
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gtgatctccg tcctggacga gcggaaccag gtggcctacg gctccaagaa cgagatcatc 900
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accgcc

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 Primer

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 Primer

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<210> 7  
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21

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&lt;211&gt; 1208

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&lt;213&gt; Triticum aestivum

&lt;400&gt; 8

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atctgcatgg gggaggcgat gaggacgac gccttcaagg tccggaccgc gtctgcggc 360
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aagctcctct tcgaggcggt ggagtactcc catgtgtgca agtacgcgtg ctctgaggaa 480
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&lt;211&gt; 322

&lt;212&gt; PRT

&lt;213&gt; Triticum aestivum

&lt;400&gt; 9

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Cys Val Asn Ser Phe Gly Asp Glu Gln Leu Ala Val Asp Met Leu Ala
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Asp Lys Leu Leu Phe Glu Ala Leu Glu Tyr Ser His Val Cys Lys Tyr
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Ala Cys Ser Glu Glu Val Pro Glu Leu Gln Asp Met Gly Gly Pro Val  
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Glu Gly Gly Phe Ser Val Ala Phe Asp Pro Leu Asp Gly Ser Ser Ile  
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Val Asp Thr Asn Phe Thr Val Gly Thr Ile Phe Gly Val Trp Pro Gly  
115 120 125

Asp Lys Leu Thr Gly Val Thr Gly Gly Asp Gln Val Ala Ala Ala Met  
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Gly Ile Tyr Gly Pro Arg Thr Thr Phe Val Val Ala Leu Lys Asp Cys  
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Pro Gly Thr His Glu Phe Leu Leu Leu Asp Glu Gly Lys Trp Gln His  
165 170 175

Val Lys Asp Thr Thr Ser Ile Gly Glu Gly Lys Met Phe Ser Pro Gly  
180 185 190

Asn Leu Arg Ala Thr Phe Asp Asn Pro Asp Tyr Asp Lys Leu Val Asn  
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Tyr Tyr Val Lys Glu Lys Tyr Thr Leu Arg Tyr Thr Gly Gly Met Val  
210 215 220

Pro Asp Val Asn Gln Ile Ile Val Lys Glu Lys Gly Ile Phe Thr Asn  
225 230 235 240

Val Thr Ser Pro Thr Ala Lys Ala Lys Leu Arg Leu Leu Phe Glu Val  
245 250 255

Ala Pro Leu Gly Phe Leu Ile Glu Lys Ala Gly Gly His Ser Ser Asp  
260 265 270

Gly Lys Gln Ser Val Leu Asp Lys Val Ile Ser Val Leu Asp Glu Arg  
275 280 285

Thr Gln Val Ala Tyr Gly Ser Lys Asn Glu Ile Ile Arg Phe Glu Glu  
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Thr Ala

&lt;210&gt; 10

&lt;211&gt; 17

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Degenerate

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## Primer

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17

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<220>  
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Primer

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35 40 45  
Arg Leu Ser Lys Ala Ser Arg Thr Ala Ala Arg Arg Ala Ala Val Ala  
50 55 60  
Ala Gln Ala Lys Ile Gly Asp Thr Leu Glu Glu Phe Leu Leu Glu Ala  
65 70 75 80  
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100 105 110  
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115 120 125  
Ala Asp Lys Leu Leu Phe Glu Ala Leu Lys Tyr Ser Gly Cys Cys Lys  
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145 150 155 160  
Gly Phe Ser Val Ala Phe Asp Pro Leu Asp Gly Ser Ser Ile Val Asp  
165 170 175

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Thr Asn Phe Ser Val Gly Thr Ile Phe Gly Val Trp Pro Gly Asp Lys  
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 Tyr Gly Pro Arg Thr Val Phe Cys Ile Ala Leu Lys Asp Ala Pro Gly  
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 Glu Thr Glu Thr Ile Gly Glu Gly Lys Met Phe Ser Pro Gly Asn Leu  
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 Arg Ala Thr Phe Asp Asn Pro Ala Tyr Glu Lys Leu Ile Ala Tyr Tyr  
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 Ser Pro Ser Thr Lys Ala Lys Leu Arg Leu Leu Phe Glu Val Ala Pro  
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&lt;211&gt; 22

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
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22

&lt;210&gt; 14

&lt;211&gt; 18

&lt;212&gt; DNA

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
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18

&lt;210&gt; 15

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Synthetic  
Primer

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&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;213&gt; Artificial Sequence

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23

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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<213> *Chlorella sorokiniana*

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35 40 45  
Arg Leu Ser Ala Lys Ala Ser Arg Thr Ala Ala Arg Arg Ala Ala Val  
50 55 60

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Ala Ala Gln Ala Lys Ile Gly Asp Thr Leu Glu Glu Phe Leu Leu Glu  
 65 70 75 80  
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 85 90 95  
 Ala Ile Arg Thr Ile Ala Tyr Lys Val Arg Thr Ala Ser Ser Gly Gly  
 100 105 110  
 Thr Ala Ser Val Asn Ser Phe Gly Asp Glu Gln Leu Ala Val Asp Leu  
 115 120 125  
 Leu Ala Asp Lys Leu Leu Phe Glu Ala Leu Lys Tyr Ser Gly Cys Cys  
 130 135 140  
 Lys Leu Ala Cys Ser Glu Glu Val Pro Glu Pro Leu Asp Leu Gly Gly  
 145 150 155 160  
 Glu Gly Phe Ser Val Ala Phe Asp Pro Leu Asp Gly Ser Ser Ile Val  
 165 170 175  
 Asp Thr Asn Phe Ser Val Gly Thr Ile Phe Gly Val Trp Pro Gly Asp  
 180 185 190  
 Lys Leu Thr Gly Ile Thr Gly Arg Gln Gln Ala Ala Gly Met Gly  
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 Ile Tyr Gly Pro Arg Thr Val Phe Cys Ile Ala Leu Lys Asp Ala Pro  
 210 215 220  
 Gly Cys His Glu Phe Gln Asp Asp Gly Lys Trp Leu His Val Lys Glu  
 225 230 235 240  
 Thr Glu Thr Ile Gly Glu Gly Lys Met Phe Ser Pro Gly Asn Leu Arg  
 245 250 255  
 Ala Thr Phe Asp Asn Pro Ala Tyr Glu Lys Leu Ile Ala Tyr Tyr Ile  
 260 265 270  
 Gly Glu Lys Tyr Thr Leu Arg Tyr Thr Gly Gly Met Val Pro Asp Val  
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 325 330 335  
 Cys Val Ser Gly Leu Asp Val Glu Val Lys Gln His Asp Gln Arg Thr  
 340 345 350  
 Gln Ile Cys Tyr Gly Ser Lys Gly Glu Val Arg Arg Phe Glu Glu Tyr  
 355 360 365

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Met	Tyr	Gly	Asn	Ser	Pro	Arg	Phe	Ser	Glu	Val	Thr	Ala
370						375					380	



## INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/US 00/13238

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/55 C12N15/82 C12N9/16

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HARRISON ELIZABETH P ET AL: "Reduced sedoheptulose-1,7-bisphosphatase levels in transgenic tobacco lead to decreased photosynthetic capacity and altered carbohydrate accumulation." PLANTA (HEIDELBERG), vol. 204, no. 1, January 1998 (1998-01), pages 27-36, XP000946358 ISSN: 0032-0935 cited in the application the whole document; esp. p.35 1. par.  -/--	1-5, 7-18,20, 21

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

21 September 2000

Date of mailing of the international search report

10/10/2000

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Authorized officer

Kania, T

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/13238

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
Y	<p>RAINES CHRISTINE A ET AL: "New insights into the structure and function of sedoheptulose-1,7-bisphosphatase; an important but neglected Calvin cycle enzyme." JOURNAL OF EXPERIMENTAL BOTANY, vol. 50, no. 330, January 1999 (1999-01), pages 1-8, XP002148063 ISSN: 0022-0957 cited in the application the whole document; esp. p.6 "Future prospects"</p>	1-5, 7-18, 20, 21
A	<p>DE 195 02 053 A (INST GENBIOLOGISCHE FORSCHUNG) 18 July 1996 (1996-07-18) the whole document</p>	1-21
A	<p>HAHN DANIELA ET AL: "The Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase is encoded by a light-regulated gene in Chlamydomonas reinhardtii." PLANT MOLECULAR BIOLOGY, vol. 36, no. 6, April 1998 (1998-04), pages 929-934, XP002148064 ISSN: 0167-4412 cited in the application the whole document</p>	1-25
A	<p>WILLINGHAM NICOLA M ET AL: "Molecular cloning of the Arabidopsis thaliana sedoheptulose-1, 7-bisphosphatase gene and expression studies in wheat and Arabidopsis thaliana." PLANT MOLECULAR BIOLOGY, vol. 26, no. 4, 1994, pages 1191-1200, XP002148065 ISSN: 0167-4412 cited in the application the whole document</p>	1-25
A	<p>MARTIN WILLIAM ET AL: "Higher-plant chloroplast and cytosolic fructose-1,6-bisphosphatase isoenzymes: Origins via duplication rather than prokaryote-eukaryote divergence." PLANT MOLECULAR BIOLOGY, vol. 32, no. 3, 1996, pages 485-491, XP002148066 ISSN: 0167-4412 cited in the application the whole document</p>	1-25
	--- -/--	

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 00/13238

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JE-GEUN YOO ET AL: "ANALYSIS OF THE CBBF GENES FROM ALCALIGENES EUTROPHUS THAT ENCODE FRUCTOSE-1,6-/SEDOHEPTULOSE-1,7-BIPHOSPHATASE" CURRENT MICROBIOLOGY, US, NEW YORK, NY, vol. 31, no. 1, 1 July 1995 (1995-07-01), pages 55-61, XP000569674 ISSN: 0343-8651 cited in the application the whole document</p>	13-25
A	<p>WO 97 12983 A (UNIV FLORIDA) 10 April 1997 (1997-04-10) the whole document</p>	1-25
P, X	<p>WO 00 03012 A (RUSSELL DOUGLAS A ; CALGENE LLC (US); MCBRIDE KEVIN E (US); NEHRA N) 20 January 2000 (2000-01-20) see claims 1-8</p>	1, 13

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/13238

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19502053 A	18-07-1996	AU 715382 B AU 4485896 A CA 2209932 A WO 9621737 A EP 0802982 A HU 9800420 A JP 11503304 T	03-02-2000 31-07-1996 18-07-1996 18-07-1996 29-10-1997 29-06-1998 26-03-1999
WO 9712983 A	10-04-1997	US 5879941 A AU 715640 B AU 7255696 A BR 9610861 A CA 2232542 A CN 1198777 A EP 0859849 A JP 11512618 T US 5985634 A	09-03-1999 10-02-2000 28-04-1997 02-03-1999 10-04-1997 11-11-1998 26-08-1998 02-11-1999 16-11-1999
WO 0003012 A	20-01-2000	NONE	

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